UNIVERSIDAD DE CÓRDOBA

DEPARTAMENTO DE GENÉTICA

TESIS DOCTORAL

"DESARROLLO Y VALIDACIÓN DE UN SISTEMA DIAGNÓSTICO RÁPIDO PARA LA DETECCIÓN DE ALTERACIONES CROMOSÓMICAS QUE PROVOCAN INFERTILIDAD EN CABALLOS BASADO EN HERRAMIENTAS MOLECULARES"

Gabriel Anaya Calvo-Rubio

Córdoba, 2017

TITULO: *DESARROLLO Y VALIDACIÓN DE UN SISTEMA DIAGNÓSTICO RÁPIDO PARA LA DETECCIÓN DE ALTERACIONES CROMOSÓMICAS QUE PROVOCAN INFERTILIDAD EN CABALLOS BASADO EN HERRAMIENTAS MOLECULARES*

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TESIS DOCTORAL

"Desarrollo y validación de un sistema diagnóstico rápido para la detección de alteraciones cromosómicas que provocan infertilidad en caballos basado en herramientas moleculares"

Gabriel Anaya Calvo-Rubio

Directores

Prof. Dr. Antonio Molina Alcalá

Prof. Dr. Sebastián Demyda Peyrás

Tutor

Prof. Dr. Miguel Moreno Millán

Córdoba, a 20 de enero de 2017

TÍTULO DE LA TESIS:

DESARROLLO Y VALIDACIÓN DE UN SISTEMA DIAGNÓSTICO RÁPIDO PARA LA DETECCIÓN DE ALTERACIONES CROMOSÓMICAS QUE PROVOCAN INFERTILIDAD EN CABALLOS BASADO EN HERRAMIENTAS MOLECULARES.

DOCTORANDO: GABRIEL ANAYA CALVO-RUBIO

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS Y EL TUTOR

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La Tesis Doctoral que se presenta está integrada por cuatro artículos publicados en revistas ISI, de gran impacto en el campo de la Ciencia Animal y un quinto artículo que actualmente se encuentra en fase de revisión en otra revista indexada. Con los resultados de la presente Tesis se ha desarrollado e implementado un panel de marcadores moleculares para la detección de las principales alteraciones cromosómicas en la especie equina relacionadas con infertilidad. La Tesis se ha estructurado en cinco capítulos:

Capítulo I: The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: A Case of sex chromosome chimerism in a Spanish Purebred colt. Los resultados de este estudio se han publicado en el trabajo:

Demyda-Peyrás, S.; Membrillo, A.; Bugno-Poniewierska, M.; Pawlina, K.; Anaya, G., and Moreno-Millán, M. The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: a case of sex chromosome chimerism in a Spanish Purebred colt. Cytogenetic and Genome Research. (2013), 141, 277-283. (IF): 1,905 (Q3). JCRC: Genetics and Heredity – Pos: 115/164 – ISSN: 1424-8581. http://dx.doi.org/10.1159/000351225

Capítulo II: The use of a novel combination of diagnostic molecular and cytogenetical approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism. Los resultados de este estudio se han publicado en el trabajo:

Demyda-Peyrás, S.; Anaya, G.; Bugno-Poniewierska, M.; Pawlina, K.; Valera, M and Moreno-Millán, M. The use of a novel combination of diagnostic molecular and cytogenetic approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism. Theriogenology. (2014), 81, (8), 1116 – 1122. (IF): 1,845 (Q1). JCRC: Veterinary Sciences– Pos: 17/129 – ISSN: 0093-691X. http://dx.doi.org/10.1016/j.theriogenology.2014.01.040

Capítulo III: Sex reversal syndrome in the horse: four new cases of feminization in individuals carrying a 64,XY SRY negative chromosomal complement. Los resultados de este estudio se han publicado en el trabajo:

Anaya G; Moreno-Millán M.; Bugno-Poniewierska, M; Pawlina K.; Membrillo A.; Molina, A and Demyda-Peyrás, S. Sex reversal syndrome in the horse: three new cases of feminization in individuals carrying a 64,XY chromosomal complement. Animal Reproduction Science. (2014), 151, 22-27. (IF): 1,581 (Q1). JCRC: Agriculture, dairy & animal science – Pos: 11/51 – ISSN: 0378-4320. http://dx.doi.org/10.1016/j.anireprosci.2014.09.020

Capítulo IV: Sex chromosomal abnormalities associated with equine infertility: Validation of a simple molecular screening tool in the Purebred Spanish Horse. Los resultados de este estudio se han publicado en el trabajo:

Anaya, G.; Molina, A.; Valera, M.; Azor, P.; Moreno Millán, M.; Peral García, P. and Demyda-Peyrás, S. Sex chromosomal abnormalities associated with equine infertility: Validation of a simple molecular screening tool in the Purebred Spanish Horse. Animal Genetics (2016). Accepted in press. (IF): 1.779 (Q1). JCRC: Agriculture Dairy & Animal Science– Pos: 8/58 – ISSN: 1365-2052. http://doi.org/10.1111/age.12543

Capítulo V: Blood chimerism in the horse is reproductively innocuous? a 15 cases report. Los resultados de este estudio se encuentran en el trabajo:

Anaya G, Valera M.; Molina A; Azor P; Moreno-Millán M.; Fernandez M. E.; Solé M.; Negro S.; y Demyda-Peyrás S. Blood chimerism in the horse is reproductively innocuous? a 28 cases report. Bajo revisión.

Asimismo, los resultados obtenidos durante el desarrollo de la Tesis se han protegido mediante la patente española titulada "MÉTODO PARA EL DIAGNÓSTICO PRECOZ DE LA INFERTILIDAD EQUINA" con número P201630755.

Por tanto, consideramos que el trabajo realizado por D. Gabriel Anaya Calvo-Rubio, bajo nuestra dirección y tutela, presenta unos elevados niveles de innovación y calidad y autorizamos su presentación y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

Córdoba, a 20 de enero de 2017

Fdo.: D. Antonio Molina Alcalá

Fdo.: D. Sebastián Demyda Peyrás

Fdo. Tutor: Miguel Moreno Millán

NOTA: esta Tesis Doctoral se presenta en parte en inglés, ya que los capítulos I a IV que la conforma han sido publicados en revistas de impacto y el capítulo V se corresponde con un artículo en fase de revisión. Para facilitar la lectura de la Tesis, al final de cada capítulo aparecen las referencias. Por esta misma razón, las referencias de la Introducción general, Capítulos y Discusión general aparecen agrupadas en el apartado REFERENCIAS BIBLIOGRÁFICAS.

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En primer lugar, me gustaría agradecer a mis directores y tutor de tesis, Antonio Molina, Sebastián Demyda y Miguel Moreno su profesionalidad, conocimiento y orientación, que permite que todo aquello en lo que invierten su tiempo y esfuerzo, tenga como resultado la mejor calidad científica. Es más fácil acercarte a la excelencia si trabajas con los mejores.

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Y sobre todo, gracias a ti Mercedes, por ser la persona que me completas, por tu comprensión, por tu amor y por Sofía.

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RESUMEN

RESUMEN

El sector equino ejerce un importante impacto en la economía de España representando el 0,51% del PIB nacional. La venta de cubriciones y semen suponen uno de los principales ingresos económicos de ésta actividad por lo que, para la rentabilidad del sector, es importante que la población de reproductores presente la máxima fertilidad posible. Las anomalías cromosómicas sexuales se consideran una de las principales causas de infertilidad en la especie equina. Entre ellas destacan aquellas que afectan al par cromosómico sexual (ECAX y ECAY), especialmente el síndrome de Turner, síndrome de sexo reverso y el quimerismo celular, que pueden constituir hasta el 95% de las alteraciones en esta especie y abarcan el mayor número de casos reportados hasta la fecha. Los animales portadores de estas patologías suelen presentar una apariencia externa y comportamental normal en edades tempranas dificultando su diagnóstico. Sin embargo, tras la pubertad, comienzan a manifestar anomalías reproductivas como esterilidad o hipoplasia gonadal. Adicionalmente el elevado coste y dificultad de las técnicas clásicas para el diagnóstico de las alteraciones cromosómicas hace que muchos de los casos queden sin diagnosticar provocando que la prevalencia real quede infraestimada.

Muy recientemente las técnicas de tipo molecular se han comenzado a utilizar como soporte a las técnicas citogenéticas en el diagnóstico de alteraciones de tipo cromosómico en humanos y animales de producción ganadera Entre éstas, los marcadores de tipo microsatélite (STR) han mostrado su utilidad de forma satisfactoria. Debido a esto, hemos desarrollado e implementado una nueva herramienta de diagnóstico molecular económica y sencilla que permite la detección de las principales alteraciones cromosómicas asociadas con infertilidad en caballos (CAPITULO 4). Para ello, se combinaron en un único panel diagnóstico, 6 marcadores STR en los cromosomas X (*LEX026*, *LEX003*, *TKY38*, *TKY270* y *UCDEQ502*) e Y (*EcaYH12*) de caballo, y se añadió el análisis de la presencia o ausencia del gen SRY con el fin de caracterizar de una manera precisa éste tipo de alteraciones genéticas. Las frecuencias de los marcadores STR utilizados se caracterizaron en una población de 271 caballos de Pura Raza Español (PRE) para determinar la eficiencia del método como herramienta diagnóstica. Los resultados

demostraron que todos los microsatélites son altamente polimórficos presentando un elevado número de alelos (Contenido de Información Polimórfica > 0,5). Basada en esta variabilidad se ha estimado una sensibilidad de 100% y una especificidad superior al 90% en el diagnóstico del síndrome de Turner, síndrome de sexo reverso y el quimerismo celular. Por último, el método molecular se validó utilizando para ello 10 animales con alteraciones cromosómicas previamente diagnosticados mediante técnicas citogenéticas con un 100% de eficiencia.

Mediante el uso de éste panel junto con otras técnicas asociadas (citogenética clásica, citogenética molecular (FISH), test de paternidad basados en STRs, etc.) hemos descrito el primer caso a nivel mundial de un quimerismo hematopoyético 64XY/63X0 en el que una de las líneas celulares era cromosómicamente anormal (CAPITULO 2). Además, durante el desarrollo de la presente tesis, hemos diagnosticado 15 animales con quimerismo sanguíneo 64XX/64XY, demostrando que son reproductivamente normales (CAPITULOS 1 y 5), y cuatro nuevos casos de yeguas sexo reverso 64,XY *SRY* negativo (CAPITULO3)

Este nuevo panel de marcadores moleculares podría utilizarse como herramienta complementaria en los programas de cría para determinar la presencia de individuos portadores de anomalías cromosómicas de una manera precoz lo que supondría un elevado ahorro de tiempo y esfuerzo para veterinarios y ganaderos.

SUMMARY

Horse production is an important activity with a considerable impact in the Spanish economy representing 0,51% of the GDP. Natural and artificial breeding's are two of the most important sales of this activity, making the fertility of the individuals one of the most important traits. It is well known that genetics, and even more chromosomal abnormalities, are a well-known cause of infertility in horses. Among these pathologies, more than 95% are related to the sex chromosome pair (ECAX and ECAY), being the syndrome of Turner, syndrome of reversal sex and cellular chimerism the most reported cases to date. The animals affected commonly shows at early ages a morphology completely normal and even a reproductive behavior according with the expectance. However, the reproductive abnormalities, such as sterility or gonadal hypoplasia are being manifested after puberty. Furthermore, chromosomal abnormalities remain undiagnosed since classical diagnostic techniques employed to date are expensive and complex, producing an underestimation of the real prevalence of this kind of genetic pathologies.

Molecular determination has been used as a complement of the cytogenetic techniques in the diagnostic of chromosomal alterations in humans and livestock animals. Among them, some reports showed that microsatellites (STR) could be used satisfactorily. Therefore, we developed and implemented a new, easy and cheap molecular diagnostic tool to determine the main infertility-associated chromosomal alterations in horses (CHAPTER 4). For this purpose, we combined, in a single diagnostic panel, 6 different STR markers in X (*LEX026*, *LEX003*, *TKY38*, *TKY270* and *UCDEQ502*) and Y (*EcaYH12*) chromosomes of the horse and the determination of the presence of the *SRY* gene to characterize comprehensively this kind of genetic diseases. All the frequencies of the STR selected were characterized in a 271 Purebreed Spanish Horse (PRE) population to determine the efficiency of the method like a diagnostic tool. Results showed that all markers were highly polymorphic, with a sizeable number of alleles (polymorphic information content > 0.5). Based on this variability, the methodology showed 100% sensitivity and specificity higher than 90% in the detection of sex chimerism and reversal syndromes and chimerisms.

Finally, the methodology was validated in individuals previously diagnosed as carriers of chromosomal abnormalities with an efficiency of 100%.

Using this panel, and several extra associated techniques (classical cytogenetics, molecular cytogenetics (FISH), parentage STR-based tests, etc.) we reported the first case in the of an hematopoietic chimerism 64XY/63X0 in which one of the cellular lines was chromosomally abnormal (CHAPTER 2). Additionally, we diagnosed in this thesis 15 animals with cellular chimerism 64XX/64XY, showing that they are reproductively normal (CHAPTER 1 and 5), and four new cases of 64,XY *SRY* negative SDS mares, the most reported in a single breed (CHAPTER 3).

Overall, this newly-developed molecular methodology could be used as a valuable complementary tool in horse breed programs to determine the presence of individuals carrying chromosomal abnormalities in early developmental stages, saving money, efforts and time to the veterinary practitioners and breeders.

INTRODUCCIÓN GENERAL

INTRODUCCIÓN GENERAL

El caballo de Pura Raza Español

El Sector Ecuestre en España genera un importante impacto económico (5.303,6 millones de €), representando el 0,51% del PIB del Estado. En la actualidad, el censo de caballos en España se sitúa en 723.496 animales que se distribuyen en 175.429 explotaciones, generando más de 61.000 puestos de trabajo. Aproximadamente un 30% de estos caballos (219,997 animales) están inscritos en libros genealógicos gestionados por diferentes asociaciones de criadores, siendo la Asociación de Criadores de Caballo de Pura Raza Español (ANCCE) la más importante de todas al agrupar aproximadamente al 85% de los mismos. En nuestro país, el principal ingreso económico de la explotación equina es la compra-venta de ejemplares, aunque, de forma muy secundaria, la venta de cubriciones o de semen puede suponer una parte importante de estos ingresos en determinadas explotaciones. El alto valor que pueden llegar a alcanzar los animales destinados a reproducción hace necesario que los mismos se encuentren libres de enfermedades y sean aptos desde el punto de vista reproductivo.

El Caballo de Pura Raza Español (PRE) no solo es la raza equina más importante de toda la Península Ibérica, sino que incluso una de las más importantes a nivel mundial (Gómez, Valera et al. 2009). Su cría comenzó en 1570 por orden del Rey Felipe II en las Caballerizas Reales de Córdoba agrupando a los mejores animales reproductores que bordeaban el rio Guadalquivir, dando origen a la Yeguada Real, posteriormente transformada en la Yeguada Nacional (Rodríguez, Sanz et al. 1992.). De la misma manera el PRE, por su calidad física, su belleza y su estirpe ha tenido gran influencia en el origen de otras razas Europeas (Lusitano, Lipizano o Paso Fino) por lo que su base genética se encuentra ampliamente distribuida en gran parte del mundo (Rodero Franganillo and Rodero Serrano 2008).

Según datos del Ministerio de Agricultura Alimentación y Medio Ambiente (http://www.magrama.gob.es/es/), el censo actual de la raza incluye 182.509 animales (55.853 yeguas

reproductoras y 33.391 sementales) distribuidos en 22.843 ganaderías en España. Además, en la actualidad existen 41.025 individuos (13.118 hembras y 8.917 machos reproductores) que se encuentran en las 8.054 explotaciones distribuidas a lo largo de 60 países. Su relevancia supera ampliamente el interés productivo con fines deportivos o de ocio puesto que estos animales son reconocidos en todo el mundo como un símbolo de la cultura y tradiciones españolas constituyéndose, de manera casi directa, en una de nuestras marcas nacionales y señas de identidad más representativas. La gestión del libro genealógico y el plan de mejora de esta raza están a cargo de la ANCCE, tanto en la península Ibérica como en el resto del mundo, habiéndose inscrito un total de 4.963 machos y 5.193 hembras en el registro de nacimientos de la sección principal del libro genealógico durante el año 2015.

Alteraciones cromosómicas en la especie equina como causa de infertilidad

La infertilidad en la especie equina se ha relacionado con numerosas causas entre las que se encuentran la edad, problemas fisiológicos y hormonales (Morley and Murray 2014, Scoggin 2015), su manejo reproductivo (Aurich 2012), así como problemas de calidad seminal y alteraciones en el comportamiento reproductivo en los machos (Madill 2002) entre otros.

Sin embargo se ha demostrado también la existencia de factores genéticos que pueden afectar a la fertilidad en esta especie (Wolc, Torzynski et al. 2009). Entre ellos, las alteraciones en la fórmula cromosómica se han descrito en numerosos trabajos como elemento asociado a la infertilidad (Chandley, Fletcher et al. 1975, Power 1990). La especie equina destaca por presentar la mayor incidencia de éste tipo de anomalías dentro del resto de especies domesticas (Villagómez, Parma et al. 2009). Tal es así, que éste tipo de anomalías se han señalado como la causa de más del 50% de las infertilidades en caballos que no poseen una explicación fisiológica visible o aparente (Infertilidad idiopática) (Lear and Bailey 2008). De entre todas ellas, el 95% en animales viables, se asocian exclusivamente a alteraciones en el par cromosómico sexual (Iannuzzi, Di Meo et al. 2004). Además, las patologías asociadas a estas alteraciones

no tienen tratamiento posible por lo que los animales afectados no pueden ser utilizados como reproductores destinándose normalmente a otros usos como pueden ser el ocio o el deporte.

Una alteración cromosómica está determinada por la variación en la estructura y/o el número de los cromosomas que tiene un individuo normal dentro de su especie (Rambags, Krijtenburg et al. 2005). La dotación cromosómica normal de un equino consiste en 31 pares de cromosomas autosómicos, identificados desde el 1 (ECA1) hasta el 31 (ECA31). Además, al igual que todas las especies superiores, cuenta con un par de cromosomas sexuales: dos cromosomas X (ECAX) en el caso de las hembras y un cromosoma X y un cromosoma Y (ECAY) en el caso de los machos. De esta manera, la fórmula cromosómica normal de la especie es 2n = 64,XX en el caso de las hembras y 2n = 64,XY en el caso de los machos (Bowling, Millon et al. 1987).

Alteraciones cromosómicas más comunes en el caballo

En la actualidad no existe un acuerdo claro entre los distintos estudios realizados sobre la incidencia real de alteraciones genéticas de origen cromosómico a nivel poblacional en caballos (Lear and McGee 2012). De hecho, el único trabajo con un número elevado y significativo fue presentado por Bugno, Słota et al. (2007) demostrando que un 2% de los individuos analizados presenta algún tipo de alteración de tipo cromosómica. Sin embargo, si se ha demostrado que son tres las alteraciones cromosómicas más comunes que provocan infertilidad en caballo: el síndrome de Turner, el síndrome de sexo reverso y el quimerismo celular, que abarcan aproximadamente el 70-80% del total de alteraciones (Lear and McGee 2012).

El síndrome de Turner, caracterizado originalmente como una monosomía del cromosoma X en los seres humanos (Ford, Jones et al. 1959), es la alteración cromosómica más comúnmente descrita en caballos (Lear, Lundquist et al. 2008, Di Meo, Neglia et al. 2009). Los animales portadores poseen una fórmula cromosómica 2n=63,X, caracterizada por la ausencia del segundo cromosoma sexual. Fenotípicamente tienen una conformación de yegua normal sin anormalidades visibles en los órganos reproductores

externos, aunque, en algunos casos los individuos pueden presentar una apariencia masculinizada. Sin embargo, los órganos reproductivos internos suelen presentar distintos grados de disgenesia gonadal asociados a casos de subfertilidad, pudiendo llegar a producir animales estériles. Además se ha demostrado que estos individuos pueden presentar en ocasiones una talla ligeramente inferior a la media e incluso la falta de comportamiento normal durante el celo (Power 1990).

Otra de las alteraciones cromosómicas más comunes en caballos es el síndrome de sexo reverso que se caracteriza por una discrepancia entre el sexo gonadal y el genético (Villagómez, Lear et al. 2011). Señalada como una de las patologías más observadas en la práctica veterinaria se caracterizó originalmente mediante la detección de dos manifestaciones diferentes: las hembras con cariotipo de macho (2n=64,XY) y los machos con cariotipo de hembra (2n=64,XX). Sin embargo, Sinclair, Berta et al. (1990) demostraron la existencia de un gen que controlaba la diferenciación sexual en los mamíferos (sex determining region; SRY). Esta secuencia, que se encuentra localizada en el cromosoma Y, codifica un factor de transcripción cuya función es la de activar una ruta metabólica molecular que involucra una serie de genes, en su mayoría localizados en cromosomas autosómicos, cuya función biológica es la supresión del desarrollo ovárico permitiendo la activación del desarrollo testicular y, por tanto, el sexo masculino. De esta manera, la ausencia del gen SRY, bien sea por la ausencia del cromosoma Y (como en las hembras) o por la delección parcial de la región que lo contiene, provoca que el desarrollo gonadal del individuo sea femenino. En la especie equina el gen SRY fue caracterizado por primera vez por Pailhoux, Cribiu et al. (1995), confirmando que el desarrollo sexual en esta especie estaba regulado por el mismo mecanismo previamente descrito. En el mismo estudio se determinó la ausencia de dicho gen en un animal fenotípicamente hembra que poseía cromosoma Y, postulando que los dos posibles casos de sexo reverso mencionados anteriormente fueran además caracterizados en base a la presencia y funcionalidad del SRY, ampliando las posibles combinaciones en la especie equina a un total de cuatro: Machos 64, XX; SRY positivo o negativo y Hembras 64, XY SRY positivo o negativo (Villagómez, Lear et al. 2011). Curiosamente, aún no se ha descrito ningún caso de macho 64,XX SRY positivo en caballos, como si ha sido descrito en los humanos (Abbas, McElreavey et al. 1993), lo que reduce la casuística a tres distintas

combinaciones, siendo las hembras 64, XY SRY negativo los casos más reportados hasta la fecha (Raudsepp, Durkin et al. 2010).

La tercera anomalía en nivel de importancia en los equinos es el quimerismo celular que aparece como consecuencia de la fusión temprana de dos embriones en sus estadios iniciales del desarrollo (quimerismo verdadero) o debido a la transferencia in útero de células sanguíneas entre fetos hermanos durante una gestación múltiple debido a una anastomosis vascular a nivel placentario (quimerismo sanguíneo). En ambos casos, si los fetos de origen son de distinto sexo, aparecerán en cada uno de ellos líneas celulares de macho y hembra. Aunque el quimerismo sanguíneo es muy común en el bovino, causando esterilidad de la hembra en el caso de gestaciones gemelares de fetos de diferente sexo (freemartinísmo; (Padula 2005)), se ha considerado hasta la fecha una condición altamente inusual en caballos, publicándose en muy pocos trabajos (Moreno-Millan, Rodero et al. 1991, Bowling, Stott et al. 1993, Bugno, Słota et al. 2007)((Miyake, Inoue et al. 1982, Juras, Raudsepp et al. 2010).

Por último, y de una manera menos frecuente, se han descrito diversos casos de mosaicismo celular que produce durante el desarrollo embrionario como consecuencia de errores en la división mitótica con lo que pueden aparecer dentro de un individuo varias poblaciones celulares (Lacadena 1996). Si bien este tipo de anomalías no son frecuentes, se han descrito asociándose, en general, a anomalías morfológicas severas en los animales portadores (Lear and McGee 2012)

Técnicas clásicas de diagnóstico citogenético

Históricamente, las alteraciones cromosomas en los animales se han diagnosticado utilizando metodologías citogenéticas convencionales basadas en la microscopía óptica (Murray, Moran et al. 1985). Estas permiten, mediante observación directa, el análisis del cariotipo del individuo y la identificación de los cromosomas involucrados en posibles anomalías mediante su morfología o mediante técnicas de bandeo. En el caso del caballo, los patrones de bandas GTG y RGB se describieron y estandarizaron de

manera definitiva en el año 1997 (Bowling, Breen et al. 1997), permitiendo identificar de manera inequívoca cada uno de los 33 cromosomas diferentes. De la misma manera, se indicaron los patrones específicos de bandeo C (desarrollado por Sumner (1972) en los seres humanos) para esta especie. Estos análisis se realizan a partir de cultivos celulares por lo que es necesaria la obtención de muestras de alta calidad (sangre con células vivas) que deben ser manipuladas y cultivadas en condiciones especiales para permitir su proliferación. Estos procedimientos precisan además de laboratorios especializados y requieren la aplicación de protocolos complejos que presentan muchos puntos críticos (Moreno-Millan, Demyda-Peyrás et al. 2012). Debido a esto, el resultado del análisis dependerá de la calidad de la muestra, la correcta aplicación y ausencia de errores del procedimiento de cultivo y la pericia de los técnicos que deberán estar altamente especializados en el uso de estas metodologías. Sin embargo, la complejidad del cariotipo del caballo (Richer, Power et al. 1990) que dificulta la correcta identificación de los cromosomas de manera individual mediante este tipo de metodologías hace que la cantidad de laboratorios especializados que pueden realizar este tipo de técnicas sea muy bajo provocando que el cariotipado del caballo sea en la práctica muy difícil de realizar (Lear and Bailey 2008).

El siguiente paso en la citogenética fue la aplicación de las técnicas de hibridación fluorescente in situ (FISH) desarrolladas originalmente por Gall and Pardue (1969), y más recientemente en el caballo por Breen, Langford et al. (1997). Esta tecnología permite la identificación individual de cualquier cromosoma, tanto en metafases como en interfases, mediante la utilización de sondas cromosoma-específicas desarrolladas expresamente para tal fin. Sin embargo, la ausencia de sondas comerciales y su elevado coste unido a ciertas restricciones inherentes a la técnica misma solo permiten la hibridación de unos pocos cromosomas a la vez, lo cual la hacen una herramienta de limitada utilidad (Pieńkowska-Schelling, Bugno et al. 2006, Bugno and SŁota 2007).

Además, a éste problema de carácter técnico, se le une un enorme problema en la detección de dichas alteraciones, en su mayoría asociadas a fallos reproductivos, debido, entre otros factores, a la ausencia de un fenotipo observable en muchos de los individuos afectados. Por lo general los animales con estas alteraciones son aparentemente normales desde el nacimiento, lo que dificulta el diagnóstico en edades

tempranas, y complica el cálculo de la prevalencia real de este tipo de patologías (Lear and Bailey 2008). En el momento de alcanzar la madurez sexual los animales comienzan a manifestar problemas reproductivos normalmente asociados a la incapacidad de los sementales para fecundar e incluso montar a las yeguas o la imposibilidad de gestar por parte de las mismas. Por tanto, hasta la detección del problema asociado a la alteración cromosómica, pueden haber pasado muchos años con el consiguiente gasto que representa para un ganadero el mantenimiento de animales no aptos para cría (300-500 € de media al mes).

Técnicas moleculares de diagnóstico de alteraciones cromosómicas

La aparición de la técnica de reacción en cadena de la polimerasa (PCR) posibilitó el análisis de diferentes tipos de marcadores moleculares mediante la amplificación de las secuencias diana (Saiki, Gelfand et al. 1988). Con ella se abrieron nuevas posibilidades y se crearon nuevas metodologías con un enfoque absolutamente distinto para el análisis de alteraciones cromosómicas. Este tipo de tecnología, que permite la detección de secuencias concretas o incluso mutaciones puntuales en el ADN, ha propiciado también el uso de los marcadores moleculares como un importante soporte en aplicaciones de tipo científicas, legales y forenses.

En los equinos, la técnicas de PCR se utilizaron en un principio para determinar la presencia del gen SRY (Pailhoux, Cribiu et al. 1995). Posteriormente, su uso ha permitido determinar el sexo genético de los animales mediante la amplificación de fragmentos específicos de diversos genes. Así, Hasegawa, Sato et al. (2000) (utilizando la amelogenina (AMEXY) y posteriormente Han, Yang et al. (2010) (utilizando las proteínas de dedos de zinc (ZFXY) demostraron que ambos genes presentan secuencias homologas en el cromosoma X e Y, pero que a su vez son de diferente tamaño en cada uno de ellos. Esta particularidad provoca que al realizar una reacción de PCR específica utilizando cebadores diseñados de forma específica se observen dos bandas diferentes en los machos (una perteneciente al ECAX y otra al ECAY) y solo una (perteneciente al ECAX) en las hembras.

Más recientemente el uso de los marcadores de tipo microsatélite, también conocidos como STR (Short Tandem Repeats), se han propuesto y utilizado como método de identificación de individuos en base a la variación en el número de repeticiones (alelos). Estos marcadores, que se localizan en los diferentes cromosomas del genoma de los individuos, segregan con una herencia de tipo mendeliana simple (Taberlet, Griffin et al. 1996) permitiendo estimar el posible genotipo de un individuo en base al de sus progenitores. Aprovechando las estas características y el hecho que el análisis del tamaño de los fragmentos amplificados por PCR se halla actualmente totalmente automatizado, el uso de este tipo de marcadores se ha extendido de manera exponencial, teniendo hoy día un sinfín de aplicaciones en la identificación animal (Bjørnstad and Røed 2002, Van De Goor, Panneman et al. 2010).

Hoy en día, el análisis por microsatélites realizado para evaluar las incompatibilidades de pedigrí entre un potro y sus progenitores consiste en un panel de 17 marcadores microsatélites, aprobados por la sociedad internacional de genética animal (ISAG) (Van De Goor, Panneman et al. 2010). Sin embargo, este panel de genotipado solo presenta un solo marcador localizado en el cromosoma X (LEX003) y ninguno en el cromosoma Y, los cromosomas más importantes en la citogenética equina (Iannuzzi and Di Berardino 2008). Anteriormente se ha pretendido su uso como herramienta de diagnóstico citogenético en el caballo generando trabajos muy limitados y aleatorios (Kakoi, Hirota et al. 2005, Juras, Raudsepp et al. 2010). Sin embargo, ninguno de estos trabajos ha planteado el uso de este tipo de marcadores de manera sistemática, desarrollando herramientas citogenéticas-moleculares específicas para tal fin.

En la actualidad los análisis citogenéticos moleculares están comenzando a realizarse mediante tecnologías de última generación basadas en el genotipado masivo. En los seres humanos la caracterización de anomalías cromosómicas se realiza mediante técnicas de hibridación genómica comparativa basada en micro-arrays (aCGH) (Shinawi and Cheung 2008), que permiten una capacidad de detección muy superior a las técnicas actualmente utilizadas (Cheung, Shaw et al. 2007). Este tipo de tecnologías se han comenzado a utilizar muy recientemente en la especie equina (Holl, Lear et al. 2013, Bugno-Poniewierska, Staroń et al. 2016). Sin embargo, y a pesar de que permiten la detección de alteraciones o microdelecciones en la totalidad de los cromosomas (autosómicos y sexuales), en el caballo

las secuencias de referencia necesarias para su utilización no se encuentran totalmente desarrolladas (Wade, Giulotto et al. 2009).

Por esta razón las nuevas metodologías moleculares desarrolladas en el transcurso de la presente investigación podrían desarrollarse y sistematizarse para realizar el diagnóstico cromosómico asociado a la fertilidad en la especie equina de una manera simple, barata, y eficiente. Esto podría lograrse mediante una herramienta específica de tipo molecular para realizar el cribado de manera rápida, fiable y económica. La aplicación precoz y de rutina de éste tipo de técnicas desde el momento del nacimiento de un potro puede suponer un elemento clave en la viabilidad de muchas ganaderías dedicadas a cría evitando el cuidado y mantenimiento de aquellos animales no aptos para la reproducción.

OBJETIVOS

OBJETIVOS

El objetivo que se persigue en la presente Tesis Doctoral es diseñar, implementar y validar un sistema diagnóstico rápido y eficaz para la detección precoz de las principales alteraciones cromosómicas que generan infertilidad en la especie equina, basado en técnicas de biología molecular, que sirva como técnica sustitutiva y/o complementaria a las técnicas clásicas de diagnóstico (técnicas citogenéticas de cariotipado y bandeo). Para la consecución de la misma, se pretende desarrollar los siguientes objetivos específicos:

1. Selección de un grupo de marcadores moleculares representativos de los cromosomas sexuales en caballo para el desarrollo de un panel diagnóstico genético específico para la detección de las enfermedades a estudiar.
2. Estimación de las capacidades de detección de dicha metodología mediante el cálculo de su sensibilidad y especificidad para cada una de las diferentes alteraciones cromosómicas relacionadas con la fertilidad previamente descritas en la especie equina.
3. Validación de la técnica en nivel estadístico utilizando como patrón resultados previamente obtenidos mediante las técnicas citogenéticas.

Por lo tanto, **la Hipótesis** que se pretende contrastar en la presente tesis es que es posible el **diseño, implementación y validación de un sistema diagnóstico rápido y eficaz para la detección precoz de las principales alteraciones cromosómicas que generan infertilidad en la especie equina, basado en técnicas de biología molecular, que sirva como técnica sustitutiva y/o complementaria a las técnicas clásicas de diagnóstico (técnicas citogenéticas de cariotipado y bandeo).**

CAPÍTULO I

Capítulo I: The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: A Case of sex chromosome chimerism in a Spanish Purebred colt.

Demyda-Peyrás S.; Membrillo A.; Bugno-Poniewierska, M; Pawlina K; **Anaya G**; and Moreno-Millán M. Cytogenetic and Genome Research. DOI: 10.1159/000351225.

Summary

Chromosomal abnormalities associated with the sexual pair are reported as a problem more common than thought in horses. Most of them remains undiagnosed due to the difficulty observed in the horse karyotype and the lack of interest of breeders and veterinarians in this type of diagnoses. Approximately 10 years ago, the Spanish Purebred Breeders Association has implemented a DNA paternity test to evaluate the pedigree of every newborn foal. All candidates who showed abnormal or uncertain results are routinely submitted to cytogenetical analysis to evaluate the presence of chromosomal abnormalities. We study the case of a foal showing three and even four different alleles in several loci in the Short Tandem Repeat (STR) based DNA parentage test. To confirm these results, filiation test was repeated using follicular hair DNA showing normal results. A complete set of conventional and molecular cytogenetic analysis were performed to determine their chromosomal complements. C-banding and *in situ* fluorescent hybridization had shown that foal presents a blood sex chimerism 64,XX/64,XY with a cellular percentage of 70/30 approximately. The use of a diagnostic approach combining routine parentage QF-PCR based STR screening tested with classical or molecular cytogenetic analysis could be a powerful tool that allows early detection of foals that will have a poor or even null reproductive performance due to chromosomal abnormalities saving time, efforts and breeders' resources.

Introduction

The Spanish Purebred Horse (SPB) is the most important equine breed reared in the Iberian Peninsula (Gómez, Valera et al. 2009). Its importance exceeds the productive interest, being recognized as a hallmark of Spanish image and traditions around the world (Valera, Molina et al. 2005). To prevent errors in the allocation of parentage, Spanish Purebred Breeders Association has implemented a mandatory DNA paternity test prior to allowing the entrance of any foal in the Association´s records. A set of 17 Short Tandem Repeat markers (STR) recommended for paternity tests and individual identification by the International Society for Animal Genetics (ISAG), is used to evaluate the pedigree of every newborn candidate. Foals showing abnormal results (three or more alleles or two or more alleles with highly unequal areas in several loci or disagreement between phenotypic and genotypic sex) are routinely submitted to chromosomal analysis to evaluate the presence of chromosomal abnormalities.

Horse karyotype was finally standardized in 1996 (Bowling, Breen et al. 1997) and it consists of 31 pairs of autosomal chromosomes and one pair of sexual chromosomes, which varies upon the sex of an animal. However, cytogenetic evaluation in horses remains very scarce, usually due to the lack of interest by owners and clinical field veterinarians in these kinds of studies (Lear and Bailey 2008) or due to the difficulty to find qualified laboratories to conduct this type of analysis (Moreno-Millan, Demyda-Peyrás et al. 2012). Normally, only infertile or sub-fertile high-valued mares or stallions that develop some kind of phenotypic alteration are submitted to cytogenetic studies. In this sense, there is a single large-scale cytogenetic screening performed to date in horses (Bugno, Słota et al. 2007). This lack of studies occurs despite the fact that primary infertility in phenotypically normal mares had repeatedly been associated with several sex chromosome abnormalities (Villagómez, Parma et al. 2009). Among horses, both true or blood chimerism reports are very unusual (Padula 2005).This may be due to twin pregnancy, mainly cause of th kind of abnormalities in mammals, is diagnosed as pathological and is normally removed at an early stage of gestation in the horse (Anne 2009). To our knowledge, previous cases of this condition were reported only in mares (Moreno-Millan, Rodero et al. 1991, Bowling, Stott et al. 1993, Bugno, Słota

et al. 2007) or dizygotic twins (Miyake, Inoue et al. 1982, Juras, Raudsepp et al. 2010). However, it has not been reported yet in a foal born in a single birth. This could be because a high number of cases remain undiagnosed. But even more, if we consider that, a high percentage of the foals which have chromosomal aberrations show no phenotypic or clinical signs until they reach adulthood (Kakoi, Hirota et al. 2005). Therefore, the aim of this study was: 1) to determine the karyotype of a SPB foal by classical and molecular cytogenetic methods; 2) to compare chromosome analysis with results of QF-PCR analysis performed on hair follicles and blood DNA and 3) to evaluate the use of complementary molecular methods in the cytogenetic analyses in the horse as a fast and reliable screening technique.

Material and methods

Animal

Samples from a registered Spanish Purebred foal were submitted to a routine blood DNA parentage test and cytogenetical analysis. Ten ml of peripheral blood were obtained by jugular venopunction using Tri-sodium ETDA Vacutainers™ for DNA isolation and sodium heparin Vacutainers™, for cell culture. At the same time, 100 hair follicles were individually collected for DNA isolation. The foal was declared as the result of a single birth without evidence of a twin pregnancy. The internal veterinary examination was not performed because the foal was too young. External revision of the animal was made without any sign of phenotypic abnormalities.

Cell Cultures and Chromosome Analysis

Chromosome preparations were made from peripheral blood lymphocyte cultures using our standard protocol (Rodero-Serrano, Demyda-Peyrás et al. 2013). Briefly, 10 ml blood samples were centrifuged at 800 g for 10 min. White cell interphase and 1 ml of autologous plasma were inoculated into 10 ml sterile tubes with 8 ml of RPMI 1640 medium supplemented with 5 µgr/ml Pokeweed Lectin, 100 IU Penicillin/ml, 100 µg/ml Streptomycin and 250 ng/ml of amphotericin B. Cultures were incubated at 38ºC

for 72 h. One hour before harvesting, 1 µgr /ml colcemid was added to each tube. After that, cells were re-centrifuged, harvested and incubated for 25 min in 0.075 M KCl hypotonic solution. Finally, cells were fixed in a cold methanol : acetic acid (3:1) solution twice and stored for 24h at 4ºC. Chromosome spreads were obtained by dropping 120µl of the cell suspension onto pre-frozen wet slides. Slides were air dried for 20 min and stained for 15 min in a 10% Giemsa water solution. Samples were assessed using a Reichert Polyvar microscope with 1250X magnification. A total of 40 analyzable metaphases (those with intact and non-overlapping chromosomes) were evaluated and counted. Sex chromosomes were assessed using C-banding, according to Sumner (1972). Percentage of 64,XY and 64,XX metaphases was assessed in 248 metaphases.

In situ hybridization

Metaphase spreads were analyzed by fluorescent *in situ* hybridization with two whole chromosome painting probes (WCPPs) specific for ECAX (*Equus caballus* chromosome X) and ECAY (*Equus caballus* chromosome Y). Probes were prepared by chromosome microdissection and DOP-PCR using our routine protocols and labeled for double color fluorescence simultaneous hybridization (Bugno, Słota et al. 2009). The X chromosome probe was DOP-labeled by biotin-16-dUTP and the Y chromosome probe by Cy3. A standard FISH protocol (Pinkel, Straume et al. 1986) with some minor modifications was applied. Labeled probes were denatured at 70ºC for 10 min. After RNAse and pepsin digestion, target metaphase spreads were denatured in a hybridization solution containing 2xSSC and 70% formamide, at 70ºC for 2.5 min. Thereafter, probes were applied onto the metaphase spreads, covered, sealed with rubber cement and hybridized overnight in a dark moisture chamber at 37ºC. The post-hybridization washes were as follows: twice in 50% formamide in 2xSSC and twice in 1xSSC at 42°C. Signals were detected and amplified using avidin-FITC, and anti-avidin antibodies. Chromosome staining was performed with DAPI. Slides were analyzed under the fluorescence microscope (Axiophot, Carl Zeiss) equipped with a digital CCD camera, driven by Lucia software (Laboratory Imaging LTD, Prague, Czech Republic).

DNA isolation and amplification

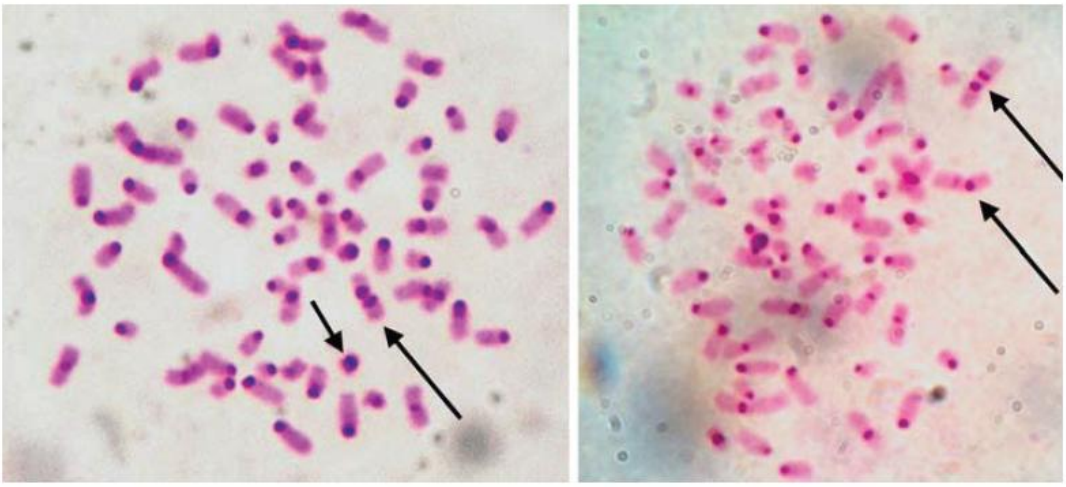
DNA was isolated separately from blood and hair follicles using QIAamp DNA mini kit (Qiagen, Carlsbad, CA) according to the manufacturer protocol. Samples were genotyped for a set of 17 STR recommended for paternity tests and individual identification by the International Society for Animal Genetics (ISAG). Microsatellites were amplified using fluorescently-labeled primers (StockMarks® for horses, PE Applied Biosystems, Foster City, CA) following the PCR conditions are given by (Dimsoski 2003) in a Mastercycler® epp gradient S thermal cycler (Eppendorf, Germany). Activation of the AmpliTaq Gold DNA polymerase was made by a single incubation for 10 min at 95 °C. Thereafter, a total of 30 cycles were performed with the following step-cycle profile: denaturation at 95 ºC for 30 sec, followed by primer annealing at 60 ºC for 30 sec, and primer extension at 72 ºC for 60 sec, with a final extension of 60 min at 72 °C. The PCR products were frozen until they were genotyped by capillary electrophoresis using an Applied Biosystems 3130 *xl* DNA sequencer. Allele sizes were determined after processing the raw data with the software package Genotyper 4.0 using a LIZ 500 bp internal size standard (Applied Biosystems).

Results

Cytogenetic analysis

Metaphases derived from blood cultures have shown two different cell types. All spreads (n=40) analyzed have shown the correct chromosomal number (2n=64) of the horse karyotype. Fourteen presumably male metaphases (35%) showed 13 pairs of bi-armed chromosomes and a long submetacentric chromosome unpaired. Twenty-six presumably female metaphases (65%) showed 14 pairs of bi-armed chromosomes. The first diagnose was a sex chromosomal blood chimerism 64,XX/64,XY. To confirm the preliminary diagnose, 248 metaphases were C-banded. As a result, 169 (68.12%) were diagnosed as metaphases with two X chromosomes, carrying a positive C-band located on the q arm and 79 (31.85%) were diagnosed as metaphases with XY chromosomes with only one chromosome showing the same pattern (Figure 1).

Figure 1: C-Banding of horse metaphases showing 64,XX and 64,XY karyotypes.

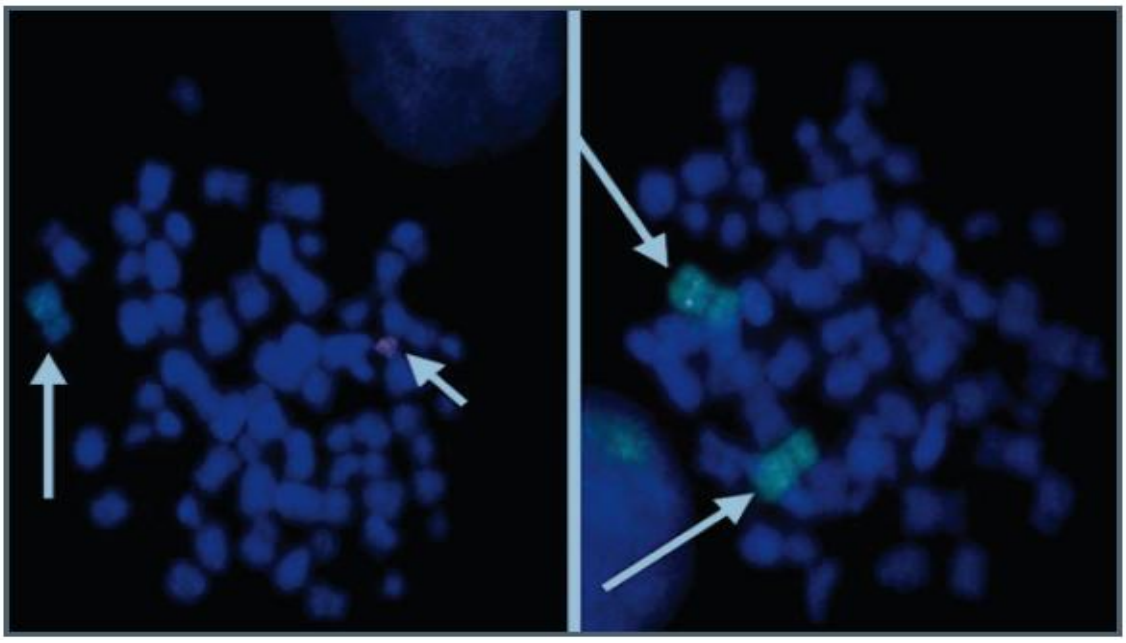


Left metaphase showing a large ECAX with 2 dark blots in the q arm (long arrow) and a small ECAY with a dark stain over the whole chromosome (short arrow). Right metaphase showing 2 ECAX with the same staining pattern (long arrows). 1,250× magnification.

In situ hybridization

Results of *in situ* hybridization agreed with those obtained using conventional cytogenetic techniques. A total of 197 hybridized metaphases were analyzed. 136 (69.03%) have shown two identical X-chromosome green signals and 61 (30.96%) have shown a large X chromosome green signal and a smaller Y-chromosome red signal (Figure 2).

Figure 2: Fluorescent *in* situ hybridization of horse metaphases showing 64,XX and 64,XY karyotypes.



A single ECAX large green signal (long arrow) and a ECAY red small signal (short arrow) are observed in the left image and two ECAX large green signals (long arrows) are observed in the right image. It is noteworthy the presence of a 64,XX (female) interphase cell in the lower-right corner on the left image showing two green signals. 1250X magnification.

STR analysis

Results obtained from microsatellite analysis from blood and hair follicles DNA are shown in table 1. Among the 17 loci tested in genomic DNA obtained from the blood sample four different alleles were observed in one loci (*ASB17*, ECA2) (Figure 3) and three alleles were observed in four loci (*AHT4*, ECA24; *AHT5*, ECA8; *ASB2*, ECA15 and *HTG4*, ECA9)**.** The remaining 12 markers were normal, with 8 heterozygous and 4 homozygous loci detected. The genotype observed in the DNA obtained from hair follicles has shown 17 loci with normal results, with 10 homozygous and 7 heterozygous loci.

Table1: Results of STR using QF-PCR of DNA obtained from blood and hair samples

Microsatellite loci	Size range, bp	Blood	Hair	ECA
AHT4	140–166	HJM	JM	24
AHT5	126–147	KMN	KN	8
ASB17	104–116	FHKM	HM	2
ASB2	237–268	KMQ	Q	15
ASB23	176–212	L	L	3
CA425	224–247	JN	JN	28
HMS1	166–178	JM	M	15
HMS2	215–236	H	H	10
HMS3	146–170	MP	MP	3
HMS6	154–170	MO	MO	4
HMS7	167–187	N	N	1
HTG10	83–110	OR	O	21
HTG4	116–137	KLM	KM	9
HTG6	74–103	JO	O	15
HTG7	114–128	NO	NO	4
LEX003	137–160	MP	P	X
VHL20	83–102	Q	Q	30

Discussion

The freemartin syndrome represents the most frequent form of intersexuality detected in several livestock species (Padula 2005). However, it is an extremely rare condition in the horse, probably due to two primary causes: Firstly, a twin pregnancy is a pathological condition in horses, representing a potentially life-threatening condition to the mare and foal (Miller and Woods 1988). Secondly, the vascular anastomosis, which occurs normally in other species, is not as commonly observed in horses. In the first case, twin pregnancies are normally detected early during pregnancy and corrected by manual reduction (Davies Morel, Newcombe et al. 2012), transvaginal ultrasound-guided aspiration (Govaere, Hoogewijs et al. 2008) and more recently by a transabdominal ultrasound-guided cardiac puncture (Sper, Whitacre et

al. 2012). Several reports have demonstrated that higher success rates are achieved when this correction is performed before the 36th day of gestation when the embryo is fixing to the endometrium (MacPherson and Reimer 2000, Govaere, Hoogewijs et al. 2008). At this stage, the cellular exchange between the fetuses is impossible since the fusion of chorionic fetal circulation has not yet occurred. In the second case, Bouters and Vandeplassche (1972) have demonstrated studying the placental circulation of 51 horse twins that only half of them has shown vascular anastomoses during pregnancy. None of those cases showed abnormal gonads or genital tracts. It was probably due to the fact that anastomoses occurred after the critical period of sex differentiation in the horse (Padula 2005).

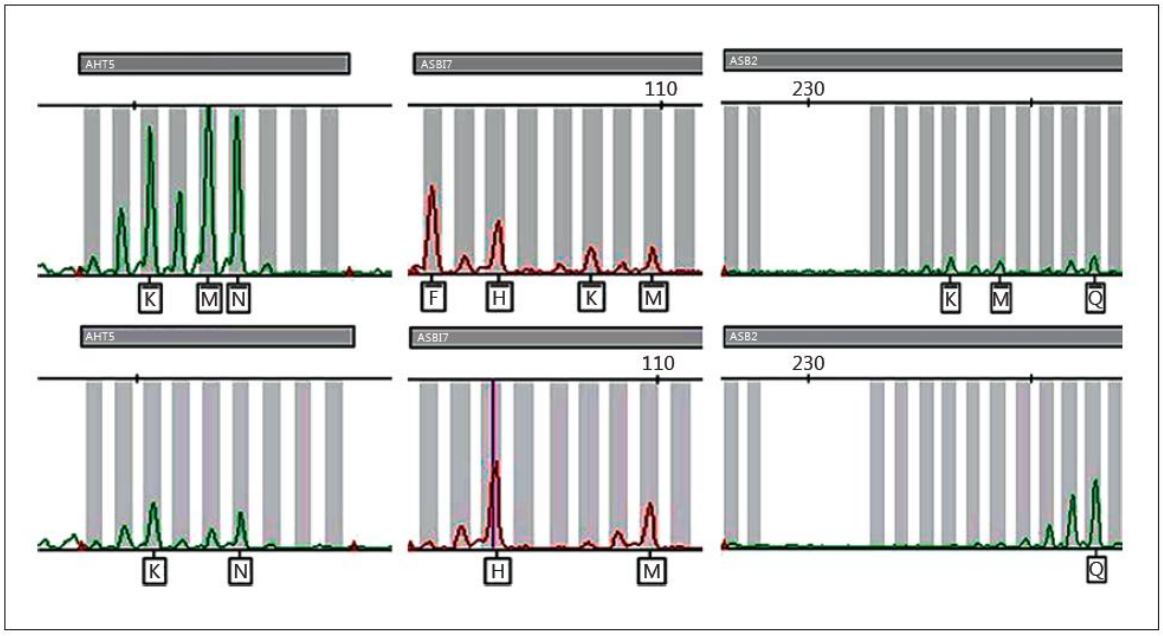
A higher than expected percentage of subfertility cases in horses may be attributed to genetically abnormal individuals (Lear and McGee 2012). However, mostly of these anomalies remains undiagnosed due to cytogenetic studies are not common in this species (Villagómez, Parma et al. 2009). Horse karyotype analysis using classical cytogenetic methods is a time-consuming difficult labor, mainly due to the elevated chromosome number and their complex morphology. To overcome this problem, the application of *in situ* hybridization could provide a fast and accurate diagnosis. However, commercial FISH probes for horse chromosomes are barely produced and its cost prevents that can be used for routine cytogenetic analysis. Conversely, the use of STR paternity tests can be considered as a valuable tool to address this issue. These tests are fewer labour-intensive diagnostic testing strategies with a better cost-benefit ratio and a wide range of commercial kits available (Donaghue, Mann et al. 2010). Today, they are increasingly used on a regular basis by breeder associations as a requirement to allow the registration of a foal in the Stud Book, due to paternal filiation miss assignments that usually occurred prior to their use. In this sense, the appearance of an abnormal allele pattern in the STRs analysis is already used as a screening technique to detect trisomies (Osborne, Trounson et al. 2005), chimerisms (Vodicka, Vrtel et al. 2004) and monosomies (Edwards and Waters 2008) in humans pregnancies. In that cases, available commercial sets of STR markers are distributed along the whole genome, existing specific markers for all human chromosomes. In our protocol, there are three STR markers in chromosome ECA15, two markers in ECA3 and ECA4 and only one marker located in 10 other different chromosomes. This means that

there are 20 chromosomes without any marker present (Table 1). Due this fact, it is possible that some cases remain undiagnosed, typically if the abnormality is located on a chromosome with no microsatellites associated.

Most of the chromosomal abnormalities reported in horses were related to the sexual chromosome pair (Villagómez, Lear et al. 2011). As a disadvantage, commercial paternity tests have only one marker (LEX003) linked to ECAX, resulting in a higher chance of misdiagnosis. In our results, a “normal” two allele pattern consistent with heterozygous loci was observed in LEX003 (*M* and *P* alleles) in blood samples, but only a homozygous *P* allele was observed in the hair samples. It only can be explained by the coexistence of two different cell lines in the same animal. Furthermore, the presence of the ECAY was not detected because there is no STR marker on it in the kit we used. To overcome this issue, Kakoi, Hirota et al. (2005) used a complementary group of STR markers linked to the sexual chromosomes (five ECAX-linked and three ECAY-linked) in horses with abnormal or doubtful results in parentage test obtaining excellent results. The possibility of misdiagnosis with several markers located in the target chromosome is virtually nil. QF-PCR is also used to diagnose the presence of a "di-allelic" trisomy by comparing marker peak area in humans (Vodicka, Vrtel et al. 2004). Two copies of a particular allele will cause a larger area than those in which one copy of the allele is present. This possibility was not tested in horses yet probably because diagnostic kits are not extremely refined and accurate as in humans. Nevertheless, ECAX monosomy (63,X0), the most common chromosomal abnormality in horses (Bugno, Ząbek et al. 2008), still cannot be confirmed without cytogenetic analysis. STR results will show a single allele in all the X-linked markers, but only karyotyping allows for the definitive determination of the absent chromosome.

In our case, we also observed "tri-allelic" loci in four different chromosomes and a "quadri-allelic" loci in ECA15 (marker *ESB17*). This combination can be only derived from two heterozygous progenitors for particular loci, that segregate two different alleles in each descendant line. For this reason, QF-PCR could be suggested as a definitive diagnostic tool for determining cellular chimerism in horses, as previously described in humans (Donaghue, Mann et al. 2010) and sheep (Martinez-Royo, Dervishi et al. 2009).

Fig. 3. Electhopherograms of microsatellite markers obtained in blood DNA (upper images) and hair follicles DNA (lower images).



The number of alleles detected in blood DNA is 3 in AHT5, 4 in ASB17 and 3 in ASB2 marker. The number of alleles observed in follicular hair DNA is 2 in AHT5 and ASB17 and only one in ASB2 marker.

Conclusion

Previous studies have demonstrated that sex chromosome abnormalities are a significant problem among equines (Kakoi, Hirota et al. 2005, Bugno, Słota et al. 2007). Actually, more and more horses with fertility disruptions are being the subject of genetic tests. We suggest that the use of a diagnostic approach combining routine parentage STR tests with classical or molecular cytogenetic analysis could be a powerful tool that allows the early detection of foals that will have poor or even nil reproductive performance due to chromosomal abnormalities. The use of this combined strategy will save time, efforts and breeders' resources.

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References

Anne SJ: Diagnosis and management off twinning in mares. In Practice 31:226-231 (2009).

Bouters R, Vandeplassche M: Twin gestation in the mare: The incidence of placental vascular anastomoses and their influence on the reproductive performance of heterosexual equine twins. J Reprod Fertil 29:149 (1972).

Bowling AT, Breen M, Chowdhary BP, Hirota K, Lear T, Millon LV, De Ponce Leon FA, Raudsepp T, Stranzinger G: Report of the third international committee for the standardization of the domestic horse karyotype, davis, ca, USA, 1996. International system for cytogenetic nomenclature of the domestic horse. Chromosome Research 5:433-443 (1997).

Bowling AT, Stott ML, Bickel L: Silent blood chimaerism in a mare confirmed by DNA marker analysis of hair bulbs. Anim Genet 24:323-324 (1993).

Bugno M, Słota E, Kościelny M: Karyotype evaluation among young horse populations in poland. Schweiz Arch Tierh 149:227-232 (2007).

Bugno M, Słota E, Pieńkowska-Schelling A, Schelling C: Identification of chromosome abnormalities in the horse using a panel of chromosome-specific painting probes generated by microdissection. Acta Vet Hung 57:369-381 (2009).

Bugno M, Ząbek T, Golonka P, Pieńkowska-Schelling A, Schelling C, Słota E: A case of an intersex horse with 63,x/64,xx/65,xx,del(y)(q?) karyotype. Cytogenet Genome Res 120:123-126 (2008).

Davies Morel MCG, Newcombe JR, Lauber M: Manual reduction of multiple embryos in the mare: The effect on subsequent pregnancy outcome. Vet J 192:322-325 (2012).

Dimsoski P: Development of a 17-plex microsatellite polymerase chain reaction kit for genotyping horses. Croatian Medical Journal 44:332-335 (2003).

Donaghue C, Mann K, Docherty Z, Mazzaschi R, Fear C, Ogilvie C: Combined qf-pcr and mlpa molecular analysis of miscarriage products: An efficient and robust alternative to karyotype analysis. Prenatal Diagnosis 30:133-137 (2010).

Edwards S, Waters JJ: Prenatal diagnosis of monosomy 18p involving a jumping translocation. Prenatal Diagnosis 28:764-766 (2008).

Gómez MD, Valera M, Molina A, Gutiérrez JP, Goyache F: Assessment of inbreeding depression for body measurements in spanish purebred (andalusian) horses. Livest Sci 122:149-155 (2009).

Govaere JLJ, Hoogewijs MK, De Schauwer C, Dewulf J, De Kruif A: Transvaginal ultrasound-guided aspiration of unilateral twin gestation in the mare. Equine Vet J 40:521-522 (2008).

Juras R, Raudsepp T, Das PJ, Conant E, Cothran EG: Xx/xy blood lymphocyte chimerism in heterosexual dizygotic twins from an american bashkir curly horse. Case report. J Equine Vet Sci 30:575-580 (2010).

Kakoi H, Hirota K, Gawahara H, Kurosawa M, Kuwajima M: Genetic diagnosis of sex chromosome aberrations in horses based on parentage test by microsatellite DNA and analysis of x- and y-linked markers. Equine Vet J 37:143-147 (2005).

Lear TL, Bailey E: Equine clinical cytogenetics: The past and future. Cytogenet Genome Res 120:42-49 (2008).

Lear TL, McGee RB: Disorders of sexual development in the domestic horse, equus caballus. Sex Dev 6:61-71 (2012).

MacPherson ML, Reimer JM: Twin reduction in the mare: Current options. Anim Reprod Sci 60-61:233-244 (2000).

Martinez-Royo A, Dervishi E, Alabart JL, Jurado JJ, Folch J, Calvo JH: Freemartinism and fecxr allele determination in replacement ewes of the rasa aragonesa sheep breed by duplex pcr. Theriogenology 72:1148-1152 (2009).

Miller A, Woods GL: Diagnosis and correction of twin pregnancy in the mare. The Veterinary clinics of North America Equine practice 4:215-220 (1988).

Miyake YI, Inoue T, Kanagawa H, Ishikawa T, Mogi K: Chromosomal analysis and blood type examination of multiple births in equine. Jpn J Vet Res 30:11-18 (1982).

Moreno-Millan M, Demyda-Peyrás S, Saleno D: Sex chromosomes abnormalities in purebred spanish horses with reproductive problems: Case reports. Bull Univ Ag Sci Vet Med Cluj-Napoca 69:1-7 (2012).

Moreno-Millan M, Rodero A, Alonso FJ, Sanz A: First report of cytogenetic studies in spanish breed horses. Genet Sel Evol 23:176-178 (1991).

Osborne EC, Trounson AO, Cram DS: A qf-pcr system to detect chromosome 13 aneuploidy from as few as ten cells. American Journal of Medical Genetics 134 A:33-38 (2005).

Padula AM: The freemartin syndrome: An update. Anim Reprod Sci 87:93-109 (2005).

Pinkel D, Straume T, Gray JW: Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. P Natl Acad Sci USA 83:2934-2938 (1986).

Rodero-Serrano E, Demyda-Peyrás S, Rodero-Franganillo A, Gonzales A, Moreno-Millán M: The chromosome translocation rob(1;29) in andalusian endangered cattle breeds Liv Sci Under Review (2013).

Sper RB, Whitacre MD, Bailey CS, Schramme AJ, Orellana DG, Ast CK, Vasgaard JM: Successful reduction of a monozygotic equine twin pregnancy via transabdominal ultrasound-guided cardiac puncture. Equine Veterinary Education 24:55-59 (2012).

Sumner AT: A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304-306 (1972).

Valera M, Molina A, Gutiérrez JP, Gómez J, Goyache F: Pedigree analysis in the andalusian horse: Population structure, genetic variability and influence of the carthusian strain. Livest Prod Sci 95:57-66 (2005).

Villagómez DAF, Lear TL, Chenier T, Lee S, McGee RB, Cahill J, Foster RA, Reyes E, St John E, King WA: Equine disorders of sexual development in 17 mares including xx, sry-negative, xy, sry-negative and xy, sry-positive genotypes. Sex Dev 5:16-25 (2011).

Villagómez DAF, Parma P, Radi O, Di Meo G, Pinton A, Iannuzzi L, King WA: Classical and molecular cytogenetics of disorders of sex development in domestic animals. Cytogenet Genome Res 126:110-131 (2009).

Vodicka R, Vrtel R, Scheinost O, Zapletalova J, Dusek L, Marie G, Santavy J: Refined quantitative fluorescent pcr of y-chromosome DNA sequences mosaics in turner's syndrome patients - alternative to real-time pcr. Journal of Biochemical and Biophysical Methods 60:151-162 (2004).

CAPÍTULO II

Capítulo II: The use of a novel combination of diagnostic molecular and cytogenetical approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism.

Demyda-Peyrás S.; **Anaya G**; Bugno-Poniewierska, M; Pawlina K; Membrillo A.; Valera M. and Moreno-Millán M. Theriogenology. DOI: 10.1016/j.theriogenology.2014.01.040

Summary

Sexual chromosomal aberrations are known to cause congenital abnormalities and unexplained infertility in horses. Most of these anomalies remain undiagnosed because of the complexity of the horse karyotype and the lack of specialized laboratories that can perform such diagnoses. On the other hand, the utilization of microsatellite markers is a technique widely spread in horse breeding, mostly due to their usage in parentage tests. We studied the usage of a novel combination of diagnostic approaches in the evaluation of a very uncommon case of chromosomal abnormalities in a Spanish Purebred colt, primarily detected using a commercial panel of short tandem repeat (STR) makers. Based on these results we performed a full cytogenetical analysis using conventional and fluorescent *in situ* hybridization techniques (FISH) techniques with individual ECAX (*Equus caballus* chromosome X) and ECAY (*Equus caballus* chromosome Y) painting probes. We also tested the presence of two genes associated with the sexual development in horses and an extra novel panel of 8 microsatellite markers specifically located in the sex chromosome pair. This is the first case reported of a leukocyte chimerism between a chromosomally normal (64,XY) and abnormal (63,X0) cell lines in horses. Our results indicate that the use of the STR markers as a screening technique and as a confirmation utilizing cytogenetic techniques can be used as a very interesting easy and non-expensive diagnostic approach to detect chromosomal abnormalities in the domestic horse.

Introduction

The association between atypical chromosome constitutions and developmental and phenotypical abnormalities has been widely described in domestic animals with the usage of clinical cytogenetics (Villagómez and Pinton 2008). In horses, these types of determinations are performed in a low number of laboratories in the entire world, probably due to the complexity of *Equus caballus* karyotype and the lack of commercially available hybridization probes to evaluate each one of their 33 different chromosomes (Lear and Bailey 2008). Because of this, some horses with abnormal karyotypes could be misdiagnosed due to the inability of conventional and molecular cytogenetic techniques to precisly determine aberations such as mosaicism or chimerism (Iannuzzi, Di Meo et al. 2004). Aditionally, the number of misdiagnoses could be higher if we consider that a great percentage of horses with this type of chromosomal aberrations are associated with normal phenotypes (Padula 2005).

On the other hand, short tandem repeat (STR) markers, a fast molecular analysis widely employed in parentage tests, could be used as a powerful screening technique to detect the existence of most of the karyotype anomalies present in certain chromosomes and even more, to detect some particular abnormalities that cannot be diagnosed using other cytogenetic techniques (Moreno-Millan, Demyda-Peyrás et al. 2012).

Currently, to perform a parentage test, the International Society for Animal Genetics (ISAG) recommends the use of a set of 17 standardized STR markers. As a disadvantage, this set of microsattellittes usually has only one marker (*LEX003*) linked to ECAX (*Equus caballus* chromosome X) and none linked to ECAY (*Equus caballus* chromosome Y). This may be problematic because most of the chromosome abnormalities reported in horses are those related to the sex chromosomes, such as the equine Turner Syndrome (X chromosome monosomy) and XY sex reversal syndrome (Lear, Lundquist et al. 2008, Di Meo, Neglia et al. 2009). For this reason, the use of extra specific microsattellite markers located in the X and Y chromosomes will make it possible to detect this kind of chromosomal disorder.

Due to this, we developed a novel diagnostic approach combining the use of cytogenetic and molecular biology techniques in order to detect horse chromosomal abnormalities in a way which is quick, simple and much cheaper [3]. In this study, we investigated the case of a Spanish Purebred horse showing an aberrant karyotype detected by using a specific set of sex chromosomes-related STR markers and molecular cytogenetic techniques. We also compared the results obtained in the analysis of different tissue samples to determine the existence of blood or true chimerism.

Material and methods

Physical examination

The colt was physically examined at the age of 14 month. It showed a normal external morphology according to the standards of the Spanish Purebred Breeders Association and it was approved to enter the official register book of the breed. External genitalia of the colt appeared to be morphologically normal without any signs of dysfunction. To date, the animal has not shown any abnormality in its behavior. Seminal parameters were not yet evaluated due to the colt sperm not being collected. However, the animal showed a normal sexual behavior in the presence of a mare in heat.

The pregnancy of which the colt was conceived was reported to the database of the Spanish Purebred Association as a single pregnancy and the foal was delivered as a single offspring. Moreover, the mother of the colt was examined by a veterinarian. It did not show any symptoms of phenotypic or physiological abnormalities.

Animal samples

Hair and blood samples were collected from the studied animal. Blood samples were obtained by jugular venopunction using Tri-sodium ETDA BD Vacutainers^{TM} (MBL, Cordoba, Spain) for DNA isolation and sodium heparin BD Vacutainers^{TM} (MBL, Cordoba, Spain) for cell culture. At the same time, 50 hair bulbs were collected for DNA isolation.

Cell Cultures and Chromosome Analysis

The chromosome slides were made from peripheral blood lymphocyte cultures using our standard protocol (Rodero-Serrano, Demyda-Peyrás et al. 2013). After centrifugation, white cells were cultured in 7 mL of RPMI 1640 medium (R4130, Sigma Aldrich, Madrid, Spain) supplemented with 5 µg/mL of Pokeweed Lectin (L9379, Sigma Aldrich) 10mL per liter of antibiotic antimicotic 100X solution (A5955, Sigma Aldrich) and 0.7 mL of autologous plasma. Cultures were incubated in 12 mL sterile culture tubes (Techno Plastic Products, Trasadingen, Switzerland), in horizontal position at 38ºC for 72 h. One hour before harvesting, 1 µg /mL of colcemid^{TM} (N-Deacetyl-N-methylcolchicine, D1925, Sigma Aldrich) was added to each tube. After that, cells were re-centrifuged, harvested and incubated for 25 min in 0.075 M KCl (Scharlau, Barcelona, Spain) hypotonic solution. Finally, cells were fixed twice in a cold methanol: acetic acid (3:1) (Qemical, Esparraguera, Spain) solution and stored for 24h at 4ºC. Chromosome spreads were obtained by cell dropping and air drying.

Giemsa stained metaphases (Rowley 1973) were assessed using a Polyvar microscope (Reichert Jung, Austria) with a x1250 magnification. A total of 100 analyzable metaphases (those with intact and non-overlapping chromosomes) were counted.

In situ hybridization

Metaphase spreads were analyzed by FISH with two whole chromosome painting probes (WCPPs) specific to ECAX and ECAY. Probes were prepared by chromosome microdissection and degenerated oligonucleotide primers (DOP) - polymerase chain reaction (PCR) amplification using our routine protocols and labeled for double color fluorescence simultaneous hybridization (Bugno, Słota et al. 2009). The ECAX probe was DOP-labeled by biotin-16- deoxyuridine triphosphate (dUTP) (Roche Applied Science, Penzberg, Germany) and the ECAY probe by Cyanine 3 dye (PerkinElmer, Waltham, USA). A standard FISH protocol (Pinkel, Straume et al. 1986) with some minor modifications was applied. The labeled probes were denatured at 70ºC for 10 min. Metaphase spreads were digested in: RNAse (10mg/mL) at room temperature for 60 min and then in 0.02% pepsin solution at 37ºC for 10 min. After

RNAse and pepsin digestion, target metaphase spreads were denatured in a hybridization solution containing 30% of 2x saline-sodium citrate (SSC) and 70% formamide (Sigma-Aldrich, St. Louis, USA) at 70ºC for 2.5 min. Thereafter, the probes were applied onto the metaphase spreads, covered, sealed with rubber cement and hybridized overnight in a dark moisture chamber at 37ºC. The post-hybridization washes were as follows: twice in 50% formamide in 2xSSC and twice in 1xSSC at 42°C. Signals were detected and amplified using avidin-Fluorescein isothiocyanate (FITC) and anti-avidin antibodies (Sigma-Aldrich, St. Louis, USA). Chromosome staining was performed with 4',6-diamidino-2-phenylindole (DAPI) (Cambio, Cambridge, UK. The slides were analyzed under the fluorescence microscope (Axiophot, Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a digital CCD camera driven by Lucia software (Laboratory Imaging LTD, Prague, Czech Republic). A 365-nm filter (UV light was used to detect the DAPI staining and a 450/490 nm filter (Blue light) was used to detect the FITC signal.

Molecular analyses

ECAX and ECAY linked genes

DNA was obtained from blood and hair follicles using the QIAamp DNA mini kit (Qiagen, Carlsbad, CA, USA) according to the manufacturer protocol. DNA from a stallion and a mare served as a positive and a negative control. DNA was amplified by PCR using primers for two genes strongly related to sex development in mammals (sex-determining region Y gene (SRY); amplified according to Hasegawa, Sato et al. (2000) and zinc finger X-chromosomal protein gene (ZFX) and zinc finger Y-chromosomal protein gene (ZFY); amplified according to Han, Yang et al. (2010)). The amplified products were assessed in a 2% agarose gel (80v, 70min run).

Microsatellite genotyping

A total of 24 STR markers were genotyped for blood and hair DNA samples. Initially, a set of 16 standard STR markers used for paternity tests in horses was assessed according to our previous studies (Demyda-Peyras, Membrillo et al. 2013). *Loci* were amplified using commercially available

fluorescently-labeled primers (StockMarks® kit for horses, PE Applied Biosystems, Foster City, CA, USA). PCR reactions were performed following the manufacturer's instructions.

Five extra X-linked (*LEX003* (Coogle, Bailey et al. 1996), *LEX0026* (Coogle, Reid et al. 1996), *TKY38* (Hirota, Tozaki et al. 2001), *TKY270* (Kakoi, Tozaki et al. 2000) and *UCEDQ502* (Eggleston-Stott, DelValle et al. 1997) and three extra Y linked (*ECAYH12*, *ECAYA16* and *ECAYM2;* (Wallner, Piumi et al. 2004)) additional markers were amplified according to Kakoi, Hirota et al. (2005). Primers for all markers were labeled using 6-carboxyfluorescein (FAM^{TM}, Sigma-Aldrich Biochemie GmbH, Hamburg, Germany) or HEX Phosphoramidite dye (HEX^{TM}, Applied Byosystems, Forster City, CA, USA) and amplified in two multiplex PCRs: one containing all the ECAY linked markers (EcaYH12, 6-FAM; EcaYA16, HEX and EcaYM2, HEX) and the second containing all the ECAX markers (*LEX003*, 6-FAM; *LEX026*, 6-FAM; *TKY38*, 6-FAM; *TKY270*, HEX and *UCDEQ502*, 6-FAM). Each PCR was performed in a 23 µL reaction mixture containing: 20–60 ng of genomic DNA, 1.5–7.5 pmol of each primer pair, 0.33 mmol/L of dNTPs, 2.5 mmol/L of $MgCl_2$, 1x PCR reaction buffer and 1.5 U of MBL Taq polymerase (Canvax Biotech, Cordoba, Spain). Samples were initially denatured at 95˚C for 10 min, followed by 33 cycles of 94˚C for 30 s, 57˚C for 1 min and 72˚C for 30 s. The final elongation step was performed at 72˚C for 10 min. Amplification products were frozen and genotyped afterwards by capillary electrophoresis using an Applied Biosystems 3130 *xl* DNA sequencer (SCAI genomics core, University of Cordoba, Spain). Allele sizes were determined after processing the raw data with the Genotyper 4.0 software package using a LIZ 500 bp internal size standard (Applied Biosystems).

Results

Cytogenetic analysis.

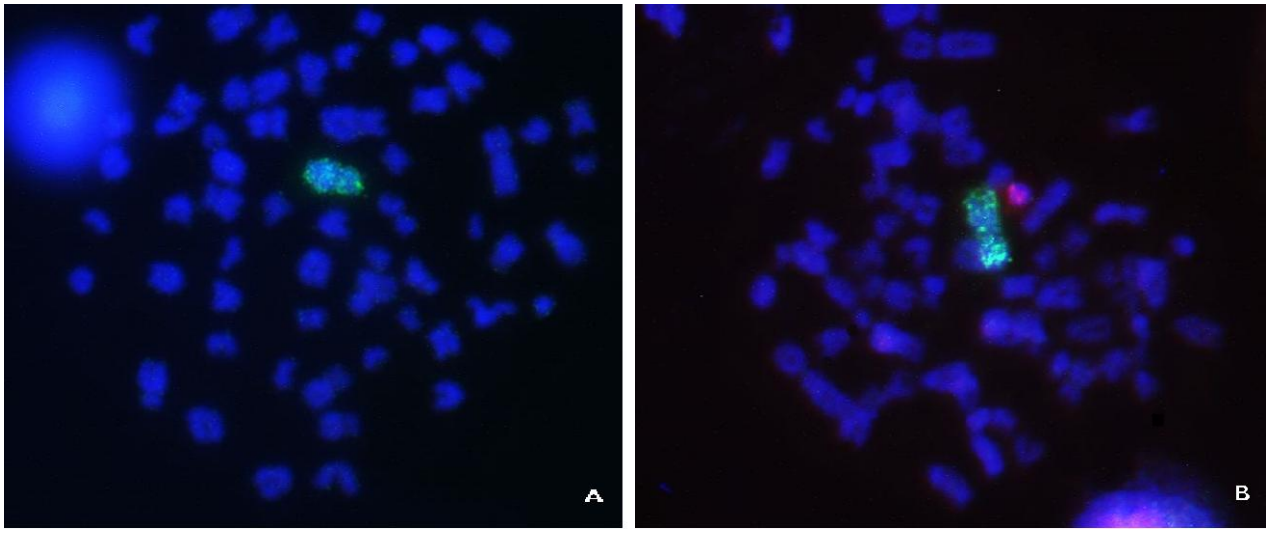
Two different karyotype lines were detected in the metaphases derived from the blood cultures. A high percentage of the spreads (98%) showed 64 chromosomes, 27 of which were diagnosed as bi-armed chromosomes. These metaphases were diagnosed as 64, XY. The remaining 2% of the analyzed spreads

showed 63 chromosomes with the same number of bi-armed chromosomes. In these metaphases, we detected the absence of one unidentified small chromosome (probably the ECAY). The first presumptive diagnose was a presence of a sex chromosomal blood mosaicism 63,X0/64,XY.

In situ hybridization

The preliminary diagnosis was confirmed by using *in situ* hybridization in a total of 96 lymphocyte spreads. The high percentages of the analyzed metaphases (95.8%) have shown a large X chromosome with a green signal and a smaller Y-chromosome with a red signal (Figure 1). The remaining metaphases (4.16%) have shown only one X-chromosome with a green signal.

Figure 1: Fluorescent in situ hybridization of horse metaphases showing 63,X0 and 64,XY karyotypes.

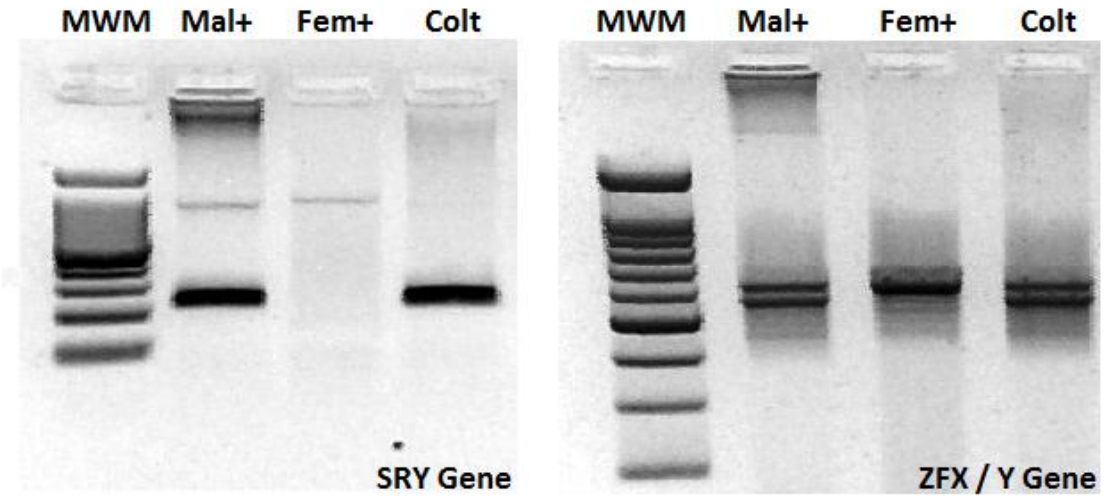


Horse metaphase spreads hybridized using two specific *Equus caballus Y chromosome (*ECAY) and *Equus caballus X chromosome* (ECAX) fluorescent labelled probes according to Bugno, Słota et al. (2009). Fig 1A: 63,X0 metaphase showing 62 autosomal chromosomes (Blue stained) and a single large green signal from the hybridization of the ECAX. Fig 1B: 64,XY metaphase showing 62 autosomal chromosomes (Blue stained), a single large green signal from the hybridization of the ECAX and a small red signal from the hybridization of ECAY. 1250X magnification.

ECAX and *ECAY* linked genes

PCR analysis performed on the DNA obtained from blood and hair samples showed the same results. ECAY specific sequences (SRY; 429bp fragment; Figure 2a and ZFY; 553bp fragment; Figure 2b) were detected in the DNA samples obtained from the studied colt and male positive control, but were not detected in the female positive control. Conversely, the specific sequence linked to ECAX (ZFX; 604 bp) was detected only in the DNA samples obtained from the studied colt and female positive control.

Figure 2: Molecular analysis of the zinc finger X and Y protein (ZFX/Y) and sex-determining region Y (SRY) genes amplified using blood and hair follicle DNA obtained from the studied Spanish Purebred colt.

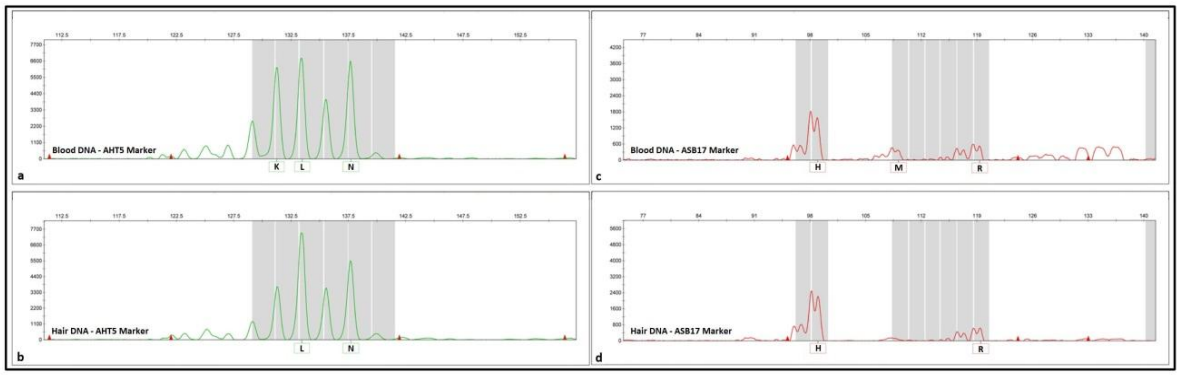


Electrophoretic gels from SRY (Fig 2a) and ZFX/Y (Fig 2b) genes. A positive SRY single fragment (429 bp) was observed in the male control and in the sample horse (Left figure). A positive ZFX fragment (604 bp) was observed in the three tested animals. The ZFY fragment (553 bp) was only observed in the male control and in the evaluated horse (right figure). MWM: molecular weight marker; Mal+: male positive control; Fem+: female positive control; Colt: studied colt

STR analysis.

The results obtained from parentage tests of STRs markers in blood and hair DNA samples are shown in Table 1. Two markers (*AHT5* and *ASB17*) showed three different alleles compatible with abnormal chromosomal complements only in DNA from blood samples (Figure 3). The remaining 14 markers were normal with 11 heterozygous and 3 homozygous loci detected. The genotype obtained from DNA acquired from hair follicles showed 16 loci with normal results (13 heterozygous and 3 homozygous loci).

Figure 3: STR (Short tandem repeat) markers from paternity test obtained from hair and blood DNA samples from the studied Spanish Purebred colt.

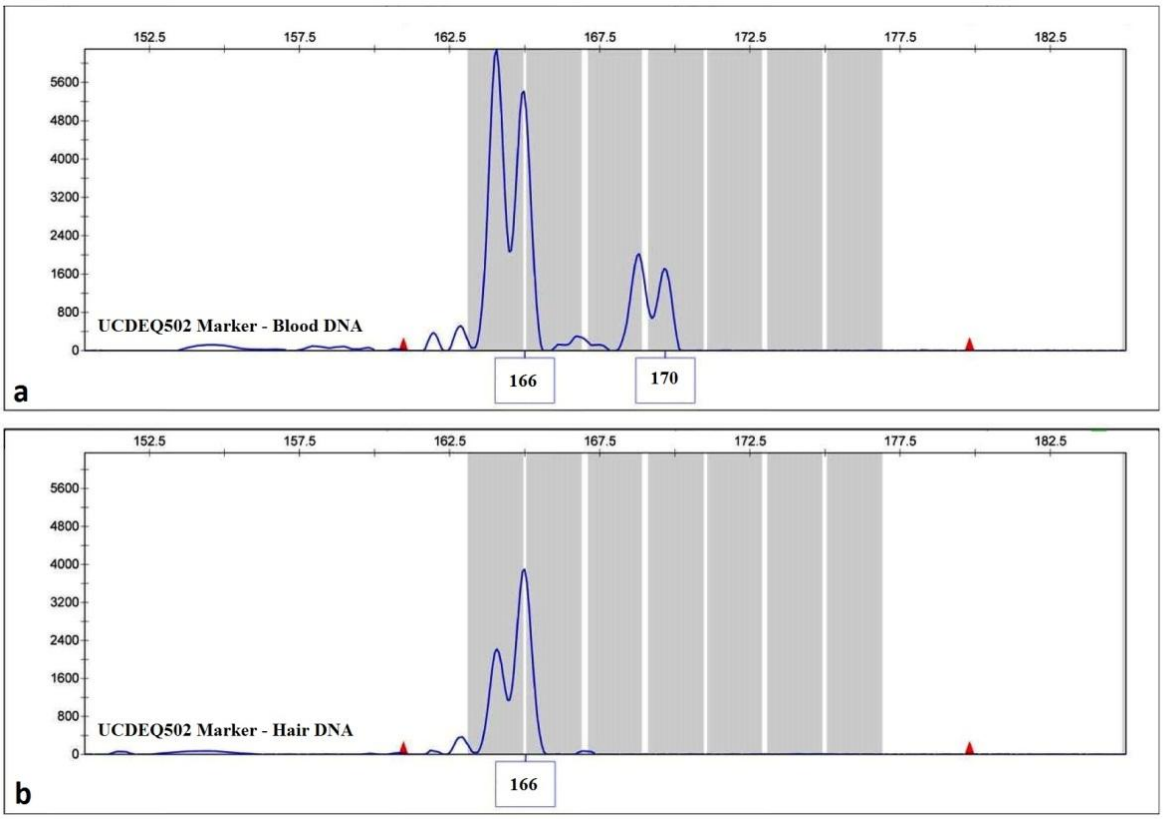


STRs electropherograms obtained from AHT5 and ASB17 markers acquired from blood and hair follicle DNA. Three alleles were observed in AHT5 blood samples (K, L and N; Fig 3a) and only two were observed in AHT5 hair samples (L and N; Fig. 3b). The same pattern was observed in ASB17 loci: Three alleles in blood samples (H, M and R; Fig 3c) and only two alleles in hair samples (H and R; Fig 3d).

Results obtained from a set of 16 standard STR markers used for paternity tests in horses, standardized by the International Society for Animal Genetics (ISAG). Loci were amplified using commercially available fluorescently-labeled primers (StockMarks® kit for horses, PE Applied Biosystems, Foster City, CA, USA). Bp: base pair; blood: DNA obtained from blood tissue; Hair: DNA obtained from hair follicles; ECA: *Equus caballus* chromosome number

The results obtained from the extra ECAX and ECAY linked microsatellites showed a similar pattern (Table 2). In DNA obtained from blood samples two of the ECAX-linked markers (*LEX026* and *UCDEQ502*) showed two different alleles (Figure 4). This result is only compatible with the existence of two X chromosomes it be originating from different cellular lines. The remaining markers (three ECAX-linked and 3 ECAY-linked markers) and all results of DNA obtained from hair follicles showed only one allele.

Figure 4: Result obtained from an expanded STR (Short tandem repeat) panel linked to the sexual chromosome pair of hair and blood DNA samples from the studied Spanish Purebred colt.



STRs electropherograms of X-linked UCDEQ502 locus acquired from blood (a) and hair (b) DNA samples. Two alleles were observed in blood DNA samples (166 and 170; Fig 4a) and only one was observed in hair DNA samples (166; Fig. 4b).

Table 1: Results of Short Tandem Repeats (STR) parentage test using DNA obtained from blood and hair samples from a Spanish purebred colt.

Microsatellite loci	Size range (bp)	Blood	Hair	ECA
AHT4	140–166	HJ	HJ	24
AHT5	126–147	KLN	LN	8
ASB17	104–116	HMR	HR	2
ASB2	237–268	KN	KN	15
ASB23	176–212	IJ	IJ	3
CA425	224–247	N	N	28
HMS1	166–178	IL	IL	15
HMS2	215–236	J	J	10
HMS3	146–170	MN	MN	3
HMS6	154–170	KN	KN	4
HMS7	167–187	LO	LO	1
HTG10	83–110	OS	OS	21
HTG4	116–137	KM	KM	9
HTG6	74–103	JO	JO	15
HTG7	114–128	O	O	4
VHL20	83–102	LQ	LQ	30

Table 2: Results of Short Tandem Repeats (STR) markers linked to the sexual chromosome pair using DNA obtained from blood and hair samples from a Spanish purebred colt.

Microsatellite loci	Size range (bp)	Blood	Hair	ECA
LEX003	194–214	210	210	X
LEX026	300–314	300/314	300	X
TKY38	105–131	129	129	X
TKY270	154–172	154	154	X
UCDEQ502	164–176	166/170	166	X
EcaYH12	95	95	95	Y
EcaYA16	154	154	154	Y
EcaYM2	118	118	118	Y

Results obtained from a set of 8 specific ECAY and ECAX linked STR markers (X-linked (*LEX003*, described by Coogle, Bailey et al. (1996) *LEX0026*, described by Coogle, Reid et al. (1996); *TKY38*, described by Hirota, Tozaki et al. (2001), *TKY270*, described by Kakoi, Tozaki et al. (2000); *UCEDQ502*, described by Eggleston-Stott, DelValle et al. (1997) and three extra Y linked (*ECAYH12*, *ECAYA16* and *ECAYM2*, described by Wallner, Piumi et al. (2004). Bp: base pair; blood: DNA obtained from blood tissue; Hair: DNA obtained from hair follicles; ECA: *Equus caballus* chromosome number

Discussion

To our knowledge, this is the first reported and proved case of a 64,X0/XY blood chimerism in horses in which the different cell lines derived from twin siblings of different sex in which one of the fetuses had also an abnormal karyotype 63,X0.

There are few reports estimating the incidence of chromosomal abnormalities in horses and only one was performed on a large population, estimating an overall rate of individuals with karyotype abnormalities at 1.6% (Bugno, Słota et al. 2007). However, in this study the rate of aberrant karyotypes was 0% for males and 3,7% for females. We suggest that these results could be influenced by the fact that chromosomal abnormalities in horses are normally linked with reproductive problems (Lear and Bailey 2008). In this sense, the number of males that are reproductively active is much lower than in mares. Furthermore, some stallions could be not diagnosed as carriers of chromosomal abnormalities because they often remain asymptomatic.

This is the fourth reported case with a 63,X0/64,XY karyotype, all of which were diagnosed as mosaicisms. The previous three reports were only diagnosed using conventional cytogenetic techniques in lymphocyte cultures (Hughes and Rommershausen-Smith 1977, Dunn, Smiley et al. 1981, Sato, Hirota et al. 2012). With these techniques, it is not possible to determine if an individual was a chimera or a mosaic or if the abnormality was present in the blood or in the whole individual. It is noteworthy that one of these previous studies speculates about the possibility of a cellular chimerism or mosaicism (Dunn, Smiley et al. 1981). However, at that time (1981), there were no available methodologies (such as PCR, FISH, etc.) to perform such determinations.

The most probable cytogenetic explanation of our results would be that the individual was carrying a cellular mosaicism in which one of the cell lines had an abnormal chromosomal complement, similar to the case previously described by Bugno, Słota et al. (2007). In both cases, the most likely hypothesis was that the two different cell lines originated from the same embryo due to an abnormal mitotic segregation. During that, the ECAY would be lost producing a different cell line with an aberrant karyotype (63,X0). However, this case is incompatible with the two different alleles observed in the *LEX026* and *UCDEQ* ECAX-linked loci and with the three different alleles observed in the autosomal loci *AHT5* and *ASB17* in the DNA obtained from blood samples. This observed microsatellite pattern and the fact that STR results from hair follicle DNA were normal can be only compatible with the mixture of blood cells belonging to two different fetuses in the maternal womb. Therefore, our case could only be derived from a blood

cellular chimerism, in which one of the embryos had additionally an abnormal 63,X0 karyotype. Thus, the combination of these two chromosomal abnormalities in a single individual is extremely rare and to our knowledge, has not been yet reported in horses.

Another interesting finding was that the foal evaluated in this study did not show any morphological or physiological abnormality in both the external and internal physical examination. This agrees with previous findings observed in horses carrying a 64,XX/64,XY chimeric karyotype. These animals are normally derived from twin heterosexual pregnancies and the fetuses and foals were completely normal (Bouters and Vandeplassche 1972, Juras, Raudsepp et al. 2010, Demyda-Peyras, Membrillo et al. 2013). This occurs because the vascular anastomosis responsible for the Freemartin syndrome in other species is developed after the sexual differentiation of the horse (Lear and McGee 2012), without without causing the typical female sterility observed in cattle (Peretti, Ciotola et al. 2008).

In the present study, both ZFX/ZFY and SRY fragments were amplified in the blood and hair DNA samples. These results (ZFY (+), ZFX (+) and SRY (+)) agree with the observed phenotypic sex of the colt, confirming that most the analyzed cells had ECAY present, presumptively derived from the 64,XY normal male embryo.

The total absence of physical abnormalities is noteworthy suggesting that there was no influence of the 4% of blood cells carrying the abnormal karyotype (63, X0) on the foal's health. These findings are totally opposite to those observed in the previously reported cases of horses carrying this type of karyotype abnormality: one horse with bilateral ovotestes, an underdeveloped penis, bilateral seminal vesicles and uterine tissue (Dunn, Smiley et al. 1981); one externally phenotypically normal mare with small, firm ovaries consisted of undifferentiated ovarian stroma (Hughes and Rommershausen-Smith 1977) and more recently a Thoroughbred colt with ambiguous external genitalia and a short penis that faced backward between its hind limbs (Sato, Hirota et al. 2012). These three cases were reported as true hermaphrodites. We suggest that the absence of phenotypical abnormalities observed in the studied foal was probably originated because, unlike the others, this was an individual carrying a blood cell

chimerism. As in the case of leukocyte chimerism 64,XX/64,XY previously described in horses, individuals carrying this chromosomal aberration have a normally developed genital organs.

Chromosomal abnormalities in livestock animals are routinely diagnosed using conventionally available cytogenetic techniques (chromosome banding), fluorescent *in situ* hybridization (FISH) (Bugno, Słota et al. 2009) and more recently comparative genomic hybridization (De Lorenzi, Rossi et al. 2012). The conventional techniques are often inaccurate in identifying individual chromosomes (Iannuzzi and Di Berardino 2008). On the contrary, FISH techniques can determine precisely chromosomes involved in an aberration; however, it is a method routinely applied only in a few laboratories around the world encountering obstacles due to the complexity of the horse karyotype (Lear and Bailey 2008). More recently, Holl, Lear et al. (2013) performed the first aneuploidy detection in horses using SNP array comparative genome hybridization (SNP-CGH). This robust diagnostic tool, increasingly used in prenatal genetic diagnostic (PGD) in humans (Harper and Sengupta 2012) is based on allelic ratios from genotyping arrays what allows an estimation of chromosome copy numbers. However, this expensive technique is currently being used commercially only in human embryos (Treff and Scott Jr 2012) and was only reported as a research tool in a very few domestic animals cases (Hornak, Jeseta et al. 2011). We, therefore, propose the use of this combination of diagnostic molecular and cytogenetical approaches that allow for a rapid screen of the presence of chromosomal abnormalities in the sex chromosomes of the horse based on the extended set of STR markers. This is a fast and accurate technique that could be utilized in a standard genetic laboratory, thus providing the possibility of being an interesting diagnostic tool in places in which other techniques may not be performed.

Conclusion

In present study the use of molecular markers widely employed for routine parentage analysis has been demonstrated to be a simple and efficient tool to detect the presence of chromosome abnormalities among horses. With the use of this method, we were able to report for the first time the ocurrence of a

chromosomal chimerism between a chromosomally normal and chromosomally aberrant cell line in horses. The use of this combined molecular technique will allow for detection and a more precise diagnosis of sex chromosome abnormalities which are known to be a severe and usually undiagnosed problem connected with reproductive failures in horses.

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Conflict of Interest

The authors does not have any conflict of interest.

References

Bouters, R. and M. Vandeplassche (1972). "Twin gestation in the mare: the incidence of placental vascular anastomoses and their influence on the reproductive performance of heterosexual equine twins." Journal of Reproduction and Fertility **29**(1): 149.

Bugno, M., E. Słota and M. Kościelny (2007). "Karyotype evaluation among young horse populations in Poland." Schweizer Archiv fur Tierheilkunde **149**(5): 227-232.

Bugno, M., E. Słota, A. Pieńkowska-Schelling and C. Schelling (2007). "Detection of equine X cromosome mosaicism in a mare using an equine X whole chromosome painting probe (WCPP) - A case report." Acta Veterinaria Hungarica **55**(2): 207-212.

Bugno, M., E. Słota, A. Pieńkowska-Schelling and C. Schelling (2009). "Identification of chromosome abnormalities in the horse using a panel of chromosome-specific painting probes generated by microdissection." Acta Veterinaria Hungarica **57**(3): 369-381.

Coogle, L., E. Bailey, R. Reid and M. Russ (1996). "Equine dinucleotide repeat polymorphisms at loci LEX002, -003, -004, -005, -007, -008, -009, -010, -011, -013 and -014." Animal genetics **27**(2): 126-127.

Coogle, L., R. Reid and E. Bailey (1996). "Equine dinucleotide repeat loci from LEX025 to LEX033." Animal Genetics **27**(4): 289-290.

De Lorenzi, L., E. Rossi, V. Genualdo, S. Gimelli, E. Lasagna, A. Perucatti, A. Iannuzzi and P. Parma (2012). "Molecular characterization of Xp chromosome deletion in a fertile cow." Sexual Development **6**(6): 298-302.

Demyda-Peyras, S., M. Bugno-Poniewierska, K. Pawlina, G. Anaya and M. Moreno-Millán (2013). "The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: A Case of sex chromosome chimerism in a Spanish Purebred colt." Cytogenetic and Genome Research **141**: 277-283.

Di Meo, G. P., G. Neglia, A. Perucatti, V. Genualdo, A. Iannuzzi, D. Crocco, D. Incarnato, G. Romano, P. Parma and L. Iannuzzi (2009). "Numerical sex chromosome aberrations and abnormal sex development in horse and sheep." Sexual Development **3**(6): 329-332.

Dunn, H. O., D. Smiley, J. R. Duncan and K. McEntee (1981). "Two equine true hermaphrodites with 64,XX/64,XY and 63,XO/64,XY chimerism." Cornell Vet **71**(2): 123-135.

Eggleston-Stott, M. L., A. DelValle, M. Bautista, S. Dileanis, E. Wictum and A. T. Bowling (1997). "Nine equine dinucleotide repeats at microsatellite loci UCDEQ136, UCDEQ405, UCDEQ412, UCDEQ425, UCDEQ437, UCDEQ467, UCDEQ487, UCDEQ502 and UCDEQ505." Animal Genetics **28**(5): 370-371.

Han, S. H., B. C. Yang, M. S. Ko, H. S. Oh and S. S. Lee (2010). "Length difference between equine ZFX and ZFY genes and its application for molecular sex determination." Journal of Assisted Reproduction and Genetics **27**(12): 725-728.

Harper, J. C. and S. B. Sengupta (2012). "Preimplantation genetic diagnosis: State of the ART 2011." Human Genetics **131**(2): 175-186.

Hasegawa, T., F. Sato, N. Ishida, Y. Fukushima and H. Mukoyama (2000). "Sex Determination by Simultaneous Amplification of Equine SRY and Amelogenin Genes." Journal of Veterinary Medical Science **62**(10): 1109-1110.

Hirota, K., T. Tozaki, S. Mashima and N. Miura (2001). "Cytogenetic assignment and genetic characterization of the horse microsatellites, TKY4-18, TKY20, TKY22-24, TKY30-41 derived from a cosmid library." Animal Genetics **32**(3): 160-162.

Holl, H. M., T. L. Lear, R. D. Nolen-Walston, J. Slack and S. A. Brooks (2013). "Detection of two equine trisomies using SNP-CGH." Mammalian Genome **24**(5-6): 252-256.

Hornak, M., M. Jeseta, P. Musilova, A. Pavlok, M. Kubelka, J. Motlik, J. Rubes and M. Anger (2011). "Frequency of aneuploidy related to age in porcine oocytes." PLoS ONE **6**(4).

Hughes, J. P. and A. T. Rommershausen-Smith (1977). "Infertility in the horse associated with chromosomal abnormalities." Australian Veterinary Journal **53**(6): 253-257.

Iannuzzi, L. and D. Di Berardino (2008). "Tools of the trade: diagnostics and research in domestic animal cytogenetics." J Appl Genet **49**(4): 357-366.

Iannuzzi, L., G. P. Di Meo, A. Perucatti, M. Spadetta, D. Incarnato, P. Parma, A. Iannuzzi, F. Ciotola, V. Peretti, G. Perrotta and R. Di Palo (2004). "Clinical, cytogenetic and molecular studies on sterile stallion and mare affected by XXY and sex reversal syndromes, respectively." Caryologia **57**(4): 400-404.

Juras, R., T. Raudsepp, P. J. Das, E. Conant and E. G. Cothran (2010). "XX/XY Blood Lymphocyte Chimerism in Heterosexual Dizygotic Twins from an American Bashkir Curly Horse. Case Report." Journal of Equine Veterinary Science **30**(10): 575-580.

Kakoi, H., K. Hirota, H. Gawahara, M. Kurosawa and M. Kuwajima (2005). "Genetic diagnosis of sex chromosome aberrations in horses based on parentage test by microsatellite DNA and analysis of X- and Y-linked markers." Equine Veterinary Journal **37**(2): 143-147.

Kakoi, H., T. Tozaki, K. Hirota, S. Mashima, M. Kurosawa and N. Miura (2000). "Ten equine microsatellite loci: TKY25, TKY26, TKY27, TKY28, TKY29, TKY267, TKY268, TKY269, TKY270 and TKY271." Animal Genetics **31**(1): 68-69.

Lear, T. L. and E. Bailey (2008). "Equine clinical cytogenetics: The past and future." Cytogenetic and Genome Research **120**(1-2): 42-49.

Lear, T. L., J. Lundquist, W. W. Zent, W. D. Fishback Jr and A. Clark (2008). "Three autosomal chromosome translocations associated with repeated early embryonic loss (REEL) in the domestic horse (Equus caballus)." Cytogenetic and Genome Research **120**(1-2): 117-122.

Lear, T. L. and R. B. McGee (2012). "Disorders of sexual development in the domestic horse, Equus caballus." Sexual Development **6**(1-3): 61-71.

Moreno-Millan, M., S. Demyda-Peyrás and D. Saleno (2012). "Sex chromosomes abnormalities in Purebred Spanish Horses with Reproductive Problems: Case Reports." Bull Univ Ag Sci Vet Med Cluj-Napoca **69**(1): 1-7.

Padula, A. M. (2005). "The freemartin syndrome: An update." Animal Reproduction Science **87**(1-2): 93-109.

Peretti, V., F. Ciotola, S. Albarella, O. Paciello, C. Dario, V. Barbieri and L. Iannuzzi (2008). "XX/XY chimerism in cattle: Clinical and cytogenetic studies." Sexual Development **2**(1): 24-30.

Pinkel, D., T. Straume and J. W. Gray (1986). "Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization." Proceedings of the National Academy of Sciences of the United States of America **83**(9): 2934-2938.

Rodero-Serrano, E., S. Demyda-Peyrás, A. González-Martinez, A. Rodero-Franganillo and M. Moreno-Millán (2013). "The rob(1;29) chromosome translocation in endangered Andalusian cattle breeds." Livestock Science **158**(1-3): 32-39.

Rowley, J. D. (1973). "A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining." Nature **243**(5405): 290-293.

Sato, F., K. Hirota, T. Tozaki, K. Ito, P. Dhakal, K. Taya, Y. Endo, H. Murase and Y. Nambo (2012). "A case of ambiguous external genitalia in a thoroughbred male horse with the 63,XO/64,XY Mosaic Karyotype." Journal of Veterinary Medical Science **74**(10): 1327-1331.

Treff, N. R. and R. T. Scott Jr (2012). "Methods for comprehensive chromosome screening of oocytes and embryos: Capabilities, limitations, and evidence of validity." Journal of Assisted Reproduction and Genetics **29**(5): 381-390.

Villagómez, D. A. F. and A. Pinton (2008). "Chromosomal abnormalities, meiotic behavior and fertility in domestic animals." Cytogenetic and Genome Research **120**(1-2): 69-80.

Wallner, B., F. Piumi, G. Brem, M. Müller and R. Achmann (2004). "Isolation of Y Chromosome-specific Microsatellites in the Horse and Cross-species Amplification in the Genus Equus." Journal of Heredity **95**(2): 158-164.

CAPÍTULO III

Capítulo III: Sex reversal syndrome in the horse: four new cases of feminization in individuals carrying a 64,XY SRY negative chromosomal complement.

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Abstract

Horses are characterized by a higher rate of chromosomal abnormalities than other species, which are mainly related to the sex chromosome pair and produce a series of different anomalies known as disorders in sexual development (DSD). We presented three Pura Raza Española (PRE) and one Menorquin (MEN) horses showing an incompatibility in their genetic and phenotypic sex. Animals were karyotyped by conventional and molecular cytogenetic analyses and characterized using genomic techniques. Although all individuals, were totally unrelated, they showed the same abnormality (64,XY *SRY* negative DSD) despite they presented an anatomically normal external mare phenotype. Therefore, we consider that this syndrome could remain undiagnosed in a higher percentage of cases since the physiological and morphological symptoms are scarce. In our study, a slightly gonadal dysgenesis was observed only in elder individuals. Interestingly this chromosomal abnormality has been previously reported less than twenty times, and never in the PRE or MEN horses. Herein we demonstrate that the use of genetic and cytogenetic diagnostic tools in veterinary practice could be an important complementary test to determine the origin of unexplained reproductive failures among horses.

Introduction

Chromosomal aberrations are more often detected in horses than in other domestic species and are normally associated with the sexual chromosome pair in more than 95% of cases (Iannuzzi et al., 2004; Lear and McGee, 2012). Furthermore, a number of animals carrying these genetic diseases show no external phenotypic changes (Demyda-Peyras et al., 2013). Due to this, a high percentage of animals

remains undiagnosed and the real prevalence of this abnormality is probably underestimated(Lear and Bailey, 2008).

Sex reversal syndrome, which is a disagreement between an individual´s genetic, gonadal and even behavioral sex, is the most common chromosomal pathology observed in veterinary practice(Villagómez et al., 2011). Currently, this pathology is classified according to the chromosomal complement detected in the individual (male or female), which should be opposite to the phenotypical sex and the presence/absence of sex determining region Y (*SRY)* gene(Mäkinen et al., 1999). According toVillagómez et al. (2011), there are four different possible conditions in horses with disorders in sexual development (DSD): 64,XX; *SRY* positive males, which have not been reported to date, 64,XX; *SRY* negative males and 64,XY mares either positive or negative for the *SRY* gene. Among them, the 64,XY *SRY* negative mares have been reported less than twenty times so far in only three recent studies performed in American (Raudsepp et al., 2010; Villagómez et al., 2011) and European horses (Bugno et al., 2003).

In this study, we analyzed for the first time four new cases of sex reversal individuals in Pura Raza Española (PRE) and Menorquín (MEN) horse breeds with conventional and molecular cytogenetic (fluorescent *in situ* hybridization - FISH) methods and genomic techniques, including specific microsatellite analysis to *Equus caballus* chromosome X- (ECAX) and Y (ECAY)-linked markers and *SRY* gene.

Material and methods

Animals

Samples from four individuals phenotypically characterized as mares, were submitted for karyotyping to our applied and molecular cytogenetic laboratory at the University of Cordoba (Spain).Three of the animals, (Case 1, 2 and 3), belonged to the PRE studbook and the fourth animal (Case 4), belonged to the MEN breed, were analyzed between 2011 and 2013. Samples were submitted to karyotyping due to the

abnormal results observed in the mandatory parentage test (performed by the breeders associations) in cases 1 and 2 or unexplained infertility in case number 3 and 4.The PRE breeders association reported that there was no parentage relationship among the PRE horses (C1, C2 and C3) up to four generations.

Physical examination and sampling procedure

All animals were examined by the official veterinary services of the respective breeder´s association. Case 1 and 2 (two fillies) were examined externally only due to their young age whereas rectal palpation and ultrasonography were performed in the two older mares (Case 3 and 4). Blood samples were obtained by jugular venopuncture using Tri-sodium ETDA BD Vacutainers^{TM} (MBL, Cordoba, Spain) for DNA isolation and sodium heparin BD Vacutainers^{TM} (MBL) for cell culture. Finally, 50 hair bulbs were aseptically collected for DNA isolation. At the time of this study, C1 was euthanized by the breeder due to a personal decision, C2 was a 13-month old and C3 and C4 were 6 and 4 years old, respectively.

Chromosome Analysis

Lymphocyte cultures were established in RPMI1640 medium for 72 h using our standard procedure (Rodero-Serrano et al., 2013). Cells were incubated horizontally in 12 mL tubes (Techno Plastic Products, Trasadingen, Switzerland), at 38ºC. After culture, cells were artificially arrested in the metaphase by incubation with colcemid (1 µg /mL, Sigma Aldrich)for 1 h; they were then treated with a hypotonic solution (25 min in 0.075M KCl) and fixed twice in a cold methanol: acetic acid (3:1) mixture. The fixative was refreshed twice a day until it became transparent. Finally, the fixed cultures were stored at a temperature of -18ºC indefinitely.

Chromosome spreads were obtained by dropping 100 µl of the fixed solution onto a clean microscopic slide and air-dried at room temperature. Chromosome number was determined in Giemsa stained slides using a Polyvar microscope (Reichert Jung, Austria) with1250 X magnification in at least 150 analyzable metaphases (those with intact and non-overlapping chromosomes).Sex chromosomes were identified and assessed using C-banding technique in at least 100 analyzable metaphases according to Sumner (1972).

In situ hybridization

Conventional chromosomal analysis was followed by *in situ* fluorescent hybridization with whole chromosome painting probes (WCPPs) specific to ECAX and ECAY. At least, 100 metaphases of each individual were analyzed. The probes were obtained by chromosome microdissection followed by non specific PCR amplification with degenerated oligonucleotide primers (DOP) according to our routine protocol (Bugno et al., 2009). The probes were labeled either indirectly (ECAX) using biotin-16-deoxyuridine triphosphate (dUTP) (Roche Applied Science, Penzberg, Germany) or directly (ECAY) using Cyanine 3 dye (Perkin Elmer, Waltham, USA). Denaturation and hybridization of the probes and slides as well as post-hybridization were carried out according to the standard protocol (Pieńkowska-Schelling et al., 2006; Bugno et al., 2007). As for the indirectly labeled ECAX probe, signals were detected and amplified using avidin-fluorescein isothiocyanate (FITC) and anti-avidin antibodies (Sigma-Aldrich). Metaphases were stained with DAPI (Cambio, Cambridge, UK). One hundred metaphases of each individual were assessed in an Axiophot fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) using Lucia software (Laboratory Imaging LTD, Prague, Czech Republic).

Molecular analyses

DNA from hair and blood of all the studied animals and from a mare and a stallion used as positive controls were isolated with the QIAamp DNA mini kit (Qiagen, Carlsbad, CA, USA). *ZFX/Y* and *SRY*, two major sex related genes located on the ECAY and ECAX were assessed by polymerase chain reaction (PCR) according to Bugno et al. (2008) and Bannasch et al. (2007). *KIT* gen was used as positive control of the PCR reaction during the *SRY* amplification according to Haase et al. (2007). The amplified products were separated in 2%agarose gels stained with ethidium bromide, by 70min at 80 V and assessed under UV light.

As a complementary study, 8 microsatellite markers (five located in the ECAX and three in the ECAY) were genotyped in both, hair and blood DNA samples, according to our procedures (Demyda-Peyrás et al., 2014). *Loci* were amplified in two multiplex PCR reactions and genotyped afterwards using an

Applied Biosystems 3130 *xl* DNA sequencer (SCAI genomics core, University of Cordoba, Spain). Allele sizes were determined with the Genotyper 4.0 software package using LIZ 500 bp internal size standard (Applied Biosystems).

Results

Physical examination

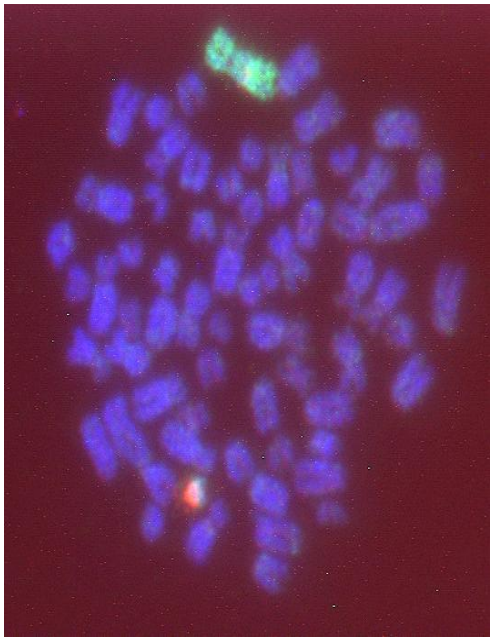
All studied animals showed a normal external morphology and a normally sized and shaped vulva, clitoris and vaginal vestibule. However, C3 and C4 showed an overall male body conformation. Reports of the owners and official veterinaries stated that the four animals did not demonstrate any female sexual behavior or cyclicity. Cases 3 and 4 were examined internally, showing a morphologically conserved uterus although slightly smaller compared to the normal dimensions of the respective breed. The ultrasonographic assessment of the ovaries showed homogenous structures with a diameter smaller than expected. There were no signs of cyclical structures like follicles or corpus lutea in either animal during one (C4) or two (C3) reproductive seasons resulting in a final diagnosis of gonadal dysgenesia.

Chromosomal assessment

The giemsa-stained karyotype showed 64 chromosomes in the analyzed metaphases of the four animals (at least 100 metaphases from each animal). C-banding also showed the presence of one ECAX and one ECAY in all analyzed metaphases. These results were compatible with a normal 64,XY male karyotype.

Results were confirmed by FISH. All metaphases showed a small red ECAY signal and a large ECAX green signal among the rest of the chromosomes counterstained in blue (Figure 1). Accordingly, the animals were diagnosed as 64,XY with a DSD.

Figure 1: Fluorescent in situ hybridization of horse metaphases showing a 64,XY karyotype.



Horse metaphase spreads hybridized using two specific *Equus caballus Y chromosome (*ECAY) and *Equus caballus X chromosome* (ECAX) fluorescent labelled probes according to Bugno et al. (2009). 64,XY metaphase showing 62 blue autosomal chromosomes, a large green signal from ECAX and a small red signal from ECAY. 1250X magnification.

Molecular characterization

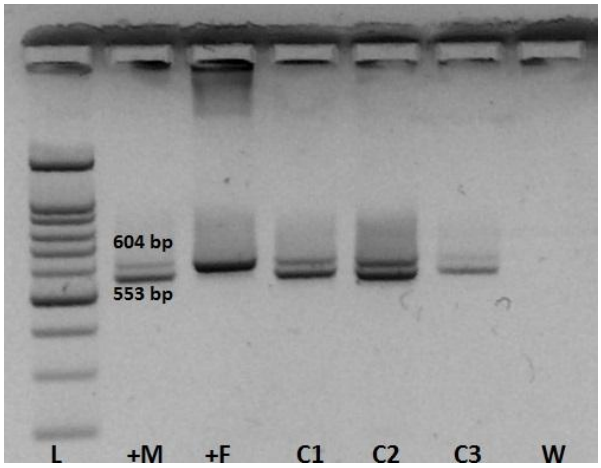
Two different genes located in the sex chromosome pair were assessed. *ZFX/Y* gene was amplified in all horses. Individuals showing the 64,XY karyotype (the four cases and the male control) showed a 604 bp specific ECAX band and a 553 bp specific ECAY band (Figure 2). Only the ECAX fragment was observed in the mare used as female control. The PCR amplification of the *SRY* gene was negative in the four cases studied and in the female control, showing an 878 bp fragment from the *KIT locus* used as competitive positive control (Figure 3). Conversely, the male positive control showed only a 242 bp band.

Table 1: Analysis of a microsatellite panel linked to the sex chromosomes of the horse in three mares with DSD

Locus	Size range (bp)	Case 1	Case 2	Case 3	Case 4	ECA
LEX003	194–214	214	206	198	198	X
LEX026	300–314	306	314	312	306	X
TKY38	105–131	129	109	129	105	X
TKY270	154–172	168	154	168	168	X
UCDEQ502	164–176	164	166	176	176	X
EcaYA16	154	154	154	154	154	Y
EcaYH12	95	95	95	95	95	Y
EcaYM2	118	118	118	118	118	Y

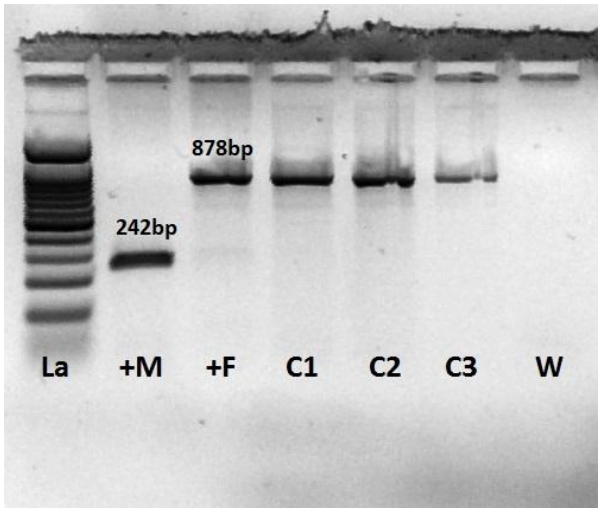
Results obtained from a specific sex-related microsatellite panel (amplified according to (Demyda-Peyrás et al., 2014)) in the four studied cases. All the animals showed a mono-allelic pattern in all the loci studied. bp: base pairs; ECA: *Equus caballus* chromosome

Figure 2: Results of the electrophoretic run of *Zinc Finger X* and *Y* genes amplification products in the studied individuals.



Two fragments (604 bp from *ZFX* and 553 bp from *ZFY*) were observed in the three studied animals (C1, C2 and C3) and in the male positive control (+M).Only one fragment (*ZFX*, 604 bp) was observed in the female positive control (+F). W: water negative control. L: molecular weight marker.

Figure 3: Results of the electrophoretic run of *SRY* and *KIT* genes amplification products in the studied individuals



A *SRY* positive fragment (242 bp) was only observed in control male (+M). A competitive 878 bp fragment (*KIT*) was observed in the three studied animals (C1, C2 and C3) and in the female positive control (+F). W: water negative control. La: molecular weight marker.

Results of microsatellite analysis are displayed in Table 1. All genotyped *loci* showed a single allele, indicating the existence of only one ECAX and ECAY. Furthermore, results were the same in both, blood and hair DNA samples, ruling out the presence of blood-cell chimerism. The lack of variability observed in the ECAY linked *loci* was remarkable, being the allele detected in each *locus* the same in all cases. Conversely, the variability observed in the ECAX-linked markers was much higher. These results confirm the presence of one ECAY and one ECAX and the preliminary diagnosis of a DSD.

Discussion

Chromosomal abnormalities are an important cause of subfertility or even sterility in horses. Among them, the DSD-linked pathologies are the most frequently detected (Villagómez et al., 2011). In our study we found four new cases of 64,XY DSD, characterized by a deletion surrounding the *SRY* region.

Previous reports have shown that 64,XY DSD animals could be classified on the basis of their morphology as exhibiting different phenotypes, from a female-like normal appearance to individuals with male-like phenotype and mixed gonadal tissue (Lear and McGee, 2012). This classification appears to be genetically correlated with the presence or absence of *SRY*, which acts as the basis to determine the type of expected anomaly. Whereas in individuals showing a normal *SRY* gene, testis tissue is usually present either as "testicle-like" structures or abdominal testicles (Villagómez et al., 2011), 64,XY *SRY* negative DSD individuals are characterized by normal external female genitalia and, in most cases, gonadal dysgenesis (Lear and McGee, 2012). These animals show great similarity with 64,X0 horses, in which their external gonads also appear like in a normal mare. Our results agree with these findings since all individuals showed normal genitalia and the C3 and C4 mares, the only one which were examined internally also showed gonadal dysgenesis and lack of estrous behavior and ciclycity.

SRY was identified as the determining trigger of the male sex in animals, by initiating the differentiation of testis rather than ovary tissue development from early pluripotential gonads (Sinclair et al., 1990). Their activation upregulates a gene cascade involving the autosomal *SOX9* gene and the steroidogenic factor 1 (SF1), leading to the anti-Müllerian hormone (AMH) production by the testis and thus, preventing the follicular development and female phenotype (De Santa Barbara et al., 1998). In humans, it is well established that a simple mutation in *SRY* could be responsible for sex reversal syndromes, among other genetic diseases (Affara et al., 1993) (Wagner et al., 1994). However, most of the studies performed in horses showing 64,XY DSD were carried out with a simple genomic technique, i.e. PCR amplification of *SRY* (Abe et al., 1999; Hasegawa et al., 2000). Using this methodology, it is possible to determine only if the amplified product (in this case only a portion of the gene) is absent or present. Due

to that, the occurrence of a specific mutation that may alter the phenotype of an individual, as was widely described in humans (Helszer et al., 2013; Xiang et al., 2013), cannot be determined.

Similarly, Ferguson-Smith (1965) demonstrated that abnormal crossing-over could be responsible for the loss of an *SRY* chromosomal section due to a misalignment produced in the post autosomal region (PAR), in which the X and Y chromosomes are paired during human meiosis. However, the detailed analysis of the horse chromosome physical map has located the *SRY* in the ECAYq14, near to centromeric region (Raudsepp et al., 2004) and the PAR on the terminal part of the long arm of the Y chromosome, together with the *AMELY* and *ZFY* genes. Due to this, it is very unlikely that the same abnormality occurs in horses. However, recent studies have shown that recombination among sex chromosomes may occur outside the pseudoautosomal regions (Rosser et al., 2009), as demonstrated in felids (Slattery et al., 2000) and suggested in horses (Raudsepp et al., 2010).

To date, most of the diagnosed horses carrying chromosomal abnormalities resulting in male-to-female sex reversal were characterized as sporadic cases (Villagómez et al., 2011). However, in previous studies performed by (Kent et al. (1986); 1988), some familiar inheritance patterns were described in Arabian sire lines. According to the PRE and MEN breeders associations, there were no reports of morphological or reproductive abnormalities among the parental and maternal lines of the studied animals, and, as mentioned before, the animals were completely unrelated. These results are concordant with more recent studies, in which the transmission of 64,XY SRY-negative DSD trough maternal or paternal lines was not demonstrated (Bugno et al., 2003; Villagómez et al., 2011). It could be possible that the lack of molecular studies in the eighties, when the inheritance of the 64,XY SRY negative DSD syndrome was proposed, could have lead to a misinterpretation of results. For instance, a recent study by Révay et al. (2012) showed that there is a hereditary component in some cases of 64,XY SRY-positive DSD. At the time of the first studies (middle eighties), there were no diagnostic methods to differentiate *SRY* positive and negative animals (Pailhoux et al., 1995), and therefore, this could lead to a misinterpretation of the results.

There are few studies regarding 64,XY *SRY* negative DSD individuals including a molecular determination of *SRY* gene (Abe et al., 1999; Mäkinen et al., 2001; Bugno et al., 2003; Villagómez et al., 2011) However, to our knowledge, a comprehensive genomic analysis in these individuals was previously performed only in 13 horses by Raudsepp et al. (2010). It is noteworthy that 11 of these animals showed the same ECAY deletion surrounding the *SRY* gene. In our preliminary results, we observed certain similarities among our cases and those previously reported (Raudsepp et al., 2010). However, we cannot determine the exact extension of the deleted sequence following the methodology reported in that manuscript because there is an identical repeated sequence located surrounding both sides of the *SRY*, and therefore, the results were inconclusive. While it is true that there is still no valid explanation for this phenomenon, we suggest that some of a specific process could lead to production of similar deletions at the same genome and in several individuals, without any particular relationship.

Conclusion

The 64,XY *SRY* negative DSD could be a prevalent genetic abnormality among the horses. However, it could remain undiagnosed mainly because animals carrying this genetic disease are anatomically normal and exhibit only symptoms of unexplained infertility or small gonads. Therefore, the use of molecular tools to diagnose this syndrome in a proper, quick and easy way could substantially improve the number of horses diagnosed, saving money and time of veterinarians and breeders.

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Conflict of Interest

The authors do not have any conflict of interest.

References

Abe, S., Y. I. Miyake, S. I. Kageyama, G. Watanabe, K. Taya and K. Kawakura (1999). "Deletion of the Sry region on the Y chromosome detected in a case of equine gonadal hypoplasia (XY female) with abnormal hormonal profiles." Equine Vet J **31**(4): 336-338.

Affara, N. A., I. J. Chalmers and M. A. Ferguson-Smith (1993). "Analysis of the SRY gene in 22 sex-reversed XY females identifies four new point mutations in the conserved DNA binding domain." Human Molecular Genetics **2**(6): 785-789.

Bannasch, D., C. Rinaldo, L. Millon, K. Latson, T. Spangler, S. Hubberty, L. Galuppo and L. Lowenstine (2007). "SRY negative 64,XX intersex phenotype in an American saddlebred horse." Veterinary Journal **173**(2): 437-439.

Bugno, M., J. Klukowska, E. Słota, M. Tischner and M. Świtoński (2003). "A sporadic case of the sex-reversed mare (64,XY; SRY-negative): Molecular and cytogenetic studies of the Y chromosome." Theriogenology **59**(7): 1597-1603.

Bugno, M., E. Słota, A. Pieńkowska-Schelling and C. Schelling (2007). "Detection of equine X cromosome mosaicism in a mare using an equine X whole chromosome painting probe (WCPP) - A case report." Acta Veterinaria Hungarica **55**(2): 207-212.

Bugno, M., E. Słota, A. Pieńkowska-Schelling and C. Schelling (2009). "Identification of chromosome abnormalities in the horse using a panel of chromosome-specific painting probes generated by microdissection." Acta Veterinaria Hungarica **57**(3): 369-381.

Bugno, M., T. Ząbek, P. Golonka, A. Pieńkowska-Schelling, C. Schelling and E. Słota (2008). "A case of an intersex horse with 63,X/64,XX/65,XX,del(Y)(q?) karyotype." Cytogenetic and Genome Research **120**(1-2): 123-126.

De Santa Barbara, P., N. Bonneaud, B. Boizet, M. Desclozeaux, B. Moniot, P. Sudbeck, G. Scherer, F. Poulat and P. Berta (1998). "Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene." Molecular and Cellular Biology **18**(11): 6653-6665.

Demyda-Peyrás, S., G. Anaya, M. Bugno-Poniewierska, K. Pawlina, A. Membrillo, M. Valera and M. Moreno-Millán (2014). "The use of a novel combination of diagnostic molecular and cytogenetic approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism." Theriogenology **81**(8): 1116-1122.

Demyda-Peyras, S., M. Bugno-Poniewierska, K. Pawlina, G. Anaya and M. Moreno-Millán (2013). "The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: A Case of sex chromosome chimerism in a Spanish Purebred colt." Cytogenetic and Genome Research **141**: 277-283.

Ferguson-Smith, M. A. (1965). "Karyotype-phenotype correlations in gonadal dysgenesis and their bearing on the pathogenesis of malformations." Journal of medical genetics **39**: 142-155.

Hasegawa, T., F. Sato, N. Ishida, Y. Fukushima and H. Mukoyama (2000). "Sex Determination by Simultaneous Amplification of Equine SRY and Amelogenin Genes." Journal of Veterinary Medical Science **62**(10): 1109-1110.

Helszer, Z., A. Dmochowska, J. Szemraj, J. Slowikowska-Hilczer, M. Wieczorek, S. Jdrzejczyk and B. Kałuzewski (2013). "A novel mutation (c. 341A>G) in the SRY gene in a 46,XY female patient with gonadal dysgenesis." Gene **526**(2): 467-470.

Iannuzzi, L., G. P. Di Meo, A. Perucatti, M. Spadetta, D. Incarnato, P. Parma, A. Iannuzzi, F. Ciotola, V. Peretti, G. Perrotta and R. Di Palo (2004). "Clinical, cytogenetic and molecular studies on sterile stallion and mare affected by XXY and sex reversal syndromes, respectively." Caryologia **57**(4): 400-404.

Kent, M. G., R. N. Shoffner, L. Buoen and A. F. Weber (1986). "XY sex-reversal syndrome in the domestic horse." Cytogenet Cell Genet **42**(1-2): 8-18.

Kent, M. G., R. N. Shoffner, A. Hunter, K. O. Elliston, W. Schroder, E. Tolley and S. S. Wachtel (1988). "XY Sex reversal syndrome in the mare: Clinical and behavioral studies, H-Y phenotype." Human Genetics **79**(4): 321-328.

Lear, T. L. and E. Bailey (2008). "Equine clinical cytogenetics: The past and future." Cytogenetic and Genome Research **120**(1-2): 42-49.

Lear, T. L. and R. B. McGee (2012). "Disorders of sexual development in the domestic horse, Equus caballus." Sexual Development **6**(1-3): 61-71.

Mäkinen, A., T. Hasegawa, M. Mäkilä and T. Katila (1999). "Infertility in two mares with XY and XXX sex chromosomes." Equine Veterinary Journal **31**(4): 346-349.

Mäkinen, A., L. Suojala, J. Niini, T. Katila, T. Tozak, Y. I. Miyake and T. Hasegawa (2001). "X chromosome detection in an XO mare using a human X paint probe, and PCR detection of SRY and amelogenin genes in 3 XY mares." Equine Veterinary Journal **33**(5): 527-530.

Pailhoux, E., E. P. Cribiu, P. Parma and C. Cotinot (1995). "Molecular analysis of an XY mare with gonadal dysgenesis." Hereditas **122**(2): 109-112.

Pieńkowska-Schelling, A., M. Bugno, M. Owczarek-Lipska, C. Schelling and E. Słota (2006). "Probe generated by Y chromosome microdissection is useful for analysing the sex chromosomes of the domestic horse." Journal of Animal and Feed Sciences **15**(2): 173-178.

Raudsepp, T., K. Durkin, T. L. Lear, P. J. Das, F. Avila, P. Kachroo and B. P. Chowdhary (2010). "Molecular heterogeneity of XY sex reversal in horses." Anim Genet **41 Suppl 2**(SUPPL. 2): 41-52.

Raudsepp, T., A. Santani, B. Wallner, S. R. Kata, C. Ren, H. B. Zhang, J. E. Womack, L. C. Skow and B. P. Chowdhary (2004). "A detailed physical map of the horse Y chromosome." Proceedings of the National Academy of Sciences of the United States of America **101**(25): 9321-9326.

Révay, T., D. A. F. Villagómez, D. Brewer, T. Chenier and W. A. King (2012). "GTG mutation in the start codon of the androgen receptor gene in a family of horses with 64,XY disorder of sex development." Sexual Development **6**(1-3): 108-116.

Rodero-Serrano, E., S. Demyda-Peyrás, A. González-Martinez, A. Rodero-Franganillo and M. Moreno-Millán (2013). "The rob(1;29) chromosome translocation in endangered Andalusian cattle breeds." Livestock Science **158**(1-3): 32-39.

Rosser, Z. H., P. Balaresque and M. A. Jobling (2009). "Gene Conversion between the X Chromosome and the Male-Specific Region of the Y Chromosome at a Translocation Hotspot." American Journal of Human Genetics **85**(1): 130-134.

Sinclair, A. H., P. Berta, M. S. Palmer, J. R. Hawkins, B. L. Griffiths, M. J. Smith, J. W. Foster, A. M. Frischauf, R. Lovell-Badge and P. N. Goodfellow (1990). "A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif." Nature **346**(6281): 240-244.

Slattery, J. P., L. Sanner-Wachter and S. J. O'Brien (2000). "Novel gene conversion between X-Y homologues located in the nonrecombining region of the Y chromosome in Felidae (Mammalia)." Proceedings of the National Academy of Sciences of the United States of America **97**(10): 5307-5312.

Sumner, A. T. (1972). "A simple technique for demonstrating centromeric heterochromatin." Experimental Cell Research **75**(1): 304-306.

Villagómez, D. A. F., T. L. Lear, T. Chenier, S. Lee, R. B. McGee, J. Cahill, R. A. Foster, E. Reyes, E. St John and W. A. King (2011). "Equine disorders of sexual development in 17 mares including XX, SRY-negative, XY, SRY-negative and XY, SRY-positive genotypes." Sexual Development **5**(1): 16-25.

Wagner, T., J. Wirth, J. Meyer, B. Zabel, M. Held, J. Zimmer, J. Pasantes, F. D. Bricarelli, J. Keutel, E. Hustert, U. Wolf, N. Tommerup, W. Schempp and G. Scherer (1994). "Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9." Cell **79**(6): 1111-1120.

Xiang, P. X., X. Dai, P. Leng, L. Liu and X. J. Hu (2013). "SRY gene-testing in the diagnosis of disorders of sex development among children." Chinese Journal of Contemporary Pediatrics **15**(7): 555-558.

CAPÍTULO IV

Capítulo IV Sex chromosomal abnormalities associated with equine infertility: Validation of a simple molecular screening tool in the Purebred Spanish Horse

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Summary

Sex chromosomal abnormalities are widely associated with reproductive problems in horses. Among them, more than 95% are related to abnormalities in the sex chromosome pair (ECAX and ECAY). However, a large proportion of these abnormalities remain undiagnosed due to the lack of an affordable diagnostic tool which allows to avoid karyotyping tests. Hereby, we developed an STR-based molecular method to determine the presence of the main sex chromosomal abnormalities in horses in a fast, cheap and reliable way. The frequency of five ECAX- *(LEX026, LEX003, TKY38, TKY270 and UCDEQ502)* and two ECAY- (*EcaYH12 and SRY*) linked markers was characterized in 261 Purebred Spanish horses (PRE) to determine the efficiency of the method as a chromosomal diagnostic tool. All the microsatellites analyzed were highly polymorphic, with a sizeable number of alleles (polymorphic information content > 0.5). Based on this variability, the methodology showed 100% sensitivity and 99.82% specificity chimerism, Turner´s syndrome and sex reversal syndromes. The method was also validated in 10 previously chromosomally aberrant diagnosed individuals with 100% efficiency. This STR screening panel is an efficient and reliable molecular-cytogenetic tool for the early detection of chromosomal abnormalities in horses which should be included in breeding programs to save money, efforts and time of veterinary practitioners and breeders.

Introduction

The Purebred Spanish horse (PRE) is the most important horse breed in the Iberian Peninsula, and one of the most important livestock production systems in Spain, with an active population of 68,971 mares and 42,308 males (Sánchez-Guerrero *et al.* 2016). PRE importance exceeds the economic benefits since it is

also recognized as an icon and hallmark of the culture and traditions of Spain. Every year, near to 10,000 new individuals are evaluated and included in the breeding program. However, and despite the fact that prevalence of sex chromosomal abnormalities reported in other polish breeds is close to 2% (Bugno *et al.* 2007), they are barely reported in these animals. The horse karyotype (2n=64) has 31 pairs of autosomal chromosomes (ECA1 to ECA31) and two sexual chromosomes (ECAX and ECAY). Its elevated complexity is determined by the presence of a large number of small acrocentric chromosomes (including ECAY), which could be easily wrongly identified, and by the fact that several metacentric chromosomes (ECA1 to ECA6) could be misleadingly taken as ECAX (Bowling et al. 1997). It was demonstrated that the prevalence of chromosomal abnormalities in horses is more common than in other domestic animals, being more than 95% of these abnormalities linked to numeric alterations in the sexual pair (Lear & McGee 2012). Historically, karyotype alterations are diagnosed by cellular cultures and classical (direct karyotyping or banding) or molecular (fluorescence *in situ* hybridization, FISH) cytogenetic techniques (Lear & Bailey 2008). However, they are difficult, expensive and time-consuming, and the number of laboratories that may perform them is small. This situation, and the fact that most of the horses with sex chromosomal pathologies do not evidence any morphological abnormality or behavioral change until adulthood has led to a higher percentage of undiagnosed cases (Lear & Bailey 2008).

Three different syndromes are responsible for more than 90% of the aberrations observed in this species. Turner´s syndrome, first described in humans in the ´50s (Ford *et al.* 1959) as the absence of a sex chromosome (2n = 63, X in horses) is the most important pathology diagnosed (Power 1987). It is usually ignored in veterinary practice since most animals bearing this disease have a normal external appearance and only a slight internal dysgenesis and irregular estrous behavior (Lear & McGee 2012). Sex reversal syndrome is the second-most reported chromosomal pathology in equines. It is characterized by a discordance among phenotype, sexual behavior and karyotype (Villagómez *et al.* 2011). Initially, this disease was reported as having two different presentations: phenotypically male animals bearing a female chromosome complement (2n = 64, XX), and vice versa (2n = 64, XY mares) (Kent *et al.* 1988). However, the characterization of the sex determining region gene *SRY* in horses by Pailhoux *et al.* (1995)

allowed to differentiate four possible sex reverse syndromes: male and female *SRY*-negative and positive individuals. Chimerism, the third most important chromosomal syndrome reported in horses, has been identified in several species and widely diagnosed as Freemartinism in cattle (Jost et al. 1972). It is derived from the exchange of blood tissue and hormones through the placental circulation between di-zygotic twin brothers. While this condition is of great importance in cattle, its prevalence in horses is very low due to the fact that twin pregnancy is a pathological condition which is normally terminated once detected (Davies Morel et al. 2012). All of them are currently diagnosed by conventional techniques, which as was mentioned previously, are difficult and time consuming and are not easily available in several countries.

Molecular markers were widely used as an effective tool for genetic identification, traceability and assessment of parentage relationships among animals (Fernández *et al.* 2013). This highly automated and fast methodology allows the detection of genetic variability among individuals and can therefore be used in scientific, forensic and legal applications. A few years ago, Kakoi *et al.* (2005) proposed that the STR-based parentage test could be used to detect sex chromosomal abnormalities in Thoroughbred horses. More recently, using a similar approach, we determined the presence of such abnormalities in two Andalusian horses with a simple DNA sample (Demyda-Peyras *et al.* 2013; Demyda-Peyrás *et al.* 2014). However, these methodologies did not include the detection of *SRY*, a gene involved in gonadal differentiation during embryonic development (Berta *et al.* 1990). This gene was demonstrated as extremely important in the differentiation among sex reversal presentations in horses (Lear & McGee 2012), and therefore, an interesting candidate to be included in a diagnostic tool.

To this end, we developed and characterized a cheap, reliable and fast diagnostic tool to detect the most important sex chromosomal abnormalities described in horses in a single multiplex polymerase chain (PCR) reaction. Furthermore, we performed a comprehensive characterization of the genetic variability of STR markers employed in an ample population of PRE horses, determining the sensitivity and specificity of our newly-developed screening method.

Material and methods

Animals

A total of 271 animals from 90 different studs belonging to the ANCCE Stud Book were analyzed. Among the total PRE population of 200,000 available specimens, 261 normal individuals (121 males and 140 females) were selected minimizing their co-ancestry values, with the aim to analyses slightly related animals. Ten extra animals previously karyotyped and diagnosed as chromosomally abnormal by our laboratory (2 chimerisms, 2 Turner´s syndrome and 6 sex reversal syndromes) were included as positive controls to validate the tool efficiency. In order to increase the accuracy of the validation, 20 extra normal animals (10 mares and 10 horses) included in the genomic characterization were also karyotyped. Blood samples were obtained by jugular venopunction using Tri-sodium ETDA Vacutainers™ for DNA extraction and Na-heparin Vacutainers™ to perform cell cultures. Hair samples (at least 50 bulbs) were also collected for DNA isolation.

Molecular analyses

DNA was obtained from blood and hair follicles using a Blood Genomic DNA Extraction Kit and a Tissue Genomic DNA Purification Kit (Canvax Biotech, Cordoba, Spain), respectively, according to the manufacturer´s protocol. Five markers located in the ECAX (*LEX003*, *UCDEQ502*, *TKY38*, *LEX026* and *TKY270*) and two markers located in the ECAY (*EcaYH12* and *SRY*) were amplified by PCR using the primer pairs previously designed by several authors (Table 1). Amplification was carried out using 23 µl of the following mixture: 20–60 ng genomic DNA, 1.5–7.5 pmol of each primer pair, 0.33 mmol/l dNTPs, 2.5 mmol/l MgCl2, 1.5 µl 10 x PCR reaction buffer, and 1.5 U Horse-Power™ Taq DNA polymerase (Canvax Biotech, Cordoba, Spain). The thermal protocol was as follows: an initial denaturation at 95˚C for 10 m; 33 cycles at 94˚C for 30 s, 57˚C for 1 m and 72˚C for 30 s, followed by 72˚C for 10 m. The amplification products were genotyped by capillary electrophoresis using an Applied Biosystems 3130 xl DNA sequencer in the Central Service for Research Support (SCAI) of the University

of Córdoba. Allele sizes were determined after processing the raw data with the software package Genemapper 4.0 using a LIZ 500 bp internal size standard (Applied Biosystems).

Table 1: Sequences of primers employed in this study.

Marker	Amplicon Size	Primers	ECA Location	References
***LEX0269* (STR)**	300-318	5´-[6-FAM]TCCAGAGTGAATGGCAAATC-3´ 5´- AAGAACTAGAACCTACAACTAGG-3´	Xp	(Coogle *et al.* 1996b)
***TKY38* (STR)**	105-141	5´-[6FAM]TAAGTATTCTCATAAACGGG-3´ 5´-GGAATAATAACAGCATCCTC-3´	Xq23	(Hirota *et al.* 2001)
***TKY270* (STR)**	156-172	5´-[HEX]CTGCTTTAGAGAAACAAACT-3´ 5´-CCATGGTGAGAAAAATGAGA-3´	X	(Kakoi *et al.* 2000)
***LEX003* (STR)**	194-216	5´-[6FAM]ACATCTAACCAGTGCTGAGACT-3´ 5´-AAGACTAGACCTACAACTAGG-3´	Xq	(Coogle *et al.* 1996a)
***UCDEQ502* (STR)**	162-176	5´-[6FAM]AGAGGGCAAAGTCAGAGCTT-3´ 5´-AGCACCTGATGCTTCTTGTT-3´	X	(Eggleston-Stott *et al.* 1997)
***EcaYH12* (STR)**	92	5´-[6FAM]CGAACAGGTGACGAAGCATC-3´ 5´-GCAGACATGCACACCAACC-3´	Y	(Wallner *et al.* 2004)
***SRY* (Gene fragment)**	249	5´-[HEX]TACCACCCTCCTCTTCAACG-3´ 5´ATCACGAGACCACACCATGA-3´	Y	(Bannasch *et al.* 2007)

ECAX STR- statistical analysis

Statistical analyses were performed using Genetix 4.0.1 (Belkhir *et al.* 1996-2004). Allele frequency (Fa) and average (Nm) and total number (Na) of alleles were determined in the entire population. Observed (Hob) and expected (Hex) heterozygosity was also calculated but only in female individuals. Allele variability (polymorphic information content, PIC) was determined for each marker in the whole population using Cervus 3.0.6. (Kalinowski *et al.* 2007). The comparison among allele frequencies was performed using the Fisher exact test with the SPSS Statistic 20 software (IBM, NY, USA). Finally, the sensitivity (proportion of positives that are correctly identified as such) and specificity (proportion of negatives that are correctly identified as such) of the diagnostic tool was determined based on the allele variability.

Cell culture and karyotyping

Twenty normal individuals (10 mares and 10 horses) were karyotyped in order to validate the molecular results. Cytogenetic analysis was performed on metaphase chromosomes obtained from lymphocytes

cultures according to Rodero-Serrano *et al.* (2013). Chromosome spreads were obtained by dropping 100 µl of the cell suspension onto wet slides. Giemsa-stained karyotypes were analyzed in a Cytovision platform (Leica, Madrid, Spain) using the *Equus caballus* standardized karyotype (Richer *et al.* 1990) and confirmed by C-banding (Sumner 1972) in at least 100 metaphases. The type and number of abnormalities detected were recorded. DNA samples from ten horses previously diagnosed with known sex chromosomal abnormalities were used as positive controls.

Results

Genetic characterization of ECAX-linked markers

Allele frequencies (Fa) of ECAX markers are show in the Table 2. All of them were highly polymorphic, with a number of alleles ranging between 6 (*TKY270*) and 11 (*LEX003*). Interestingly, Na was higher in males in three ECAX-linked markers, being seven different alleles only represented in males (202 and 216 in *LEX003*; 302, 304 and 310 in *LEX026* and 172 and 176 in *UCDEQ502*). On the contrary, five different alleles (318 in *LEX026*; 127 and 139 in *TKY38* and 170 in *UCDEQ502*) were represented only in mares. Three markers (*LEX003, LEX026* and *UCDEQ502*) showed a similar Fa between sexes, whereas two of them (*TKY270* and *TKY38*) had a different Fa between sexes ($P<0.05$). Finally, Nm per marker (8.4) also demonstrated a high genetic variability in the population analyzed.

Expected (Hex) and observed (Hob) heterozygosity's and PIC´s are shown in Table 3. *LEX003* showed the highest Hex (0.802) and Hob (0.761) values, whereas *TKY38* (0.679) and *UCDEQ502* (0.575) showed the lowest. PIC was higher than 0.50 in all the ECAX-linked markers analyzed.

Table 3: Observed (Hob), expected heterozygosity (Hex) and Polymorphic information contain (PIC) of the ECAX microsatellites calculated in the entire population.

STR	H_{ob}	H_{ex}	PIC
LEX003	0.761	0.802	0.796
LEX026	0.659	0.714	0.682
TKY270	0.698	0.694	0.663
TKY38	0.674	0.679	0.665
UCDEQ502	0.575	0.682	0.631

Table 2: Allelic Frequencies (AF) obtained in the five ECAX microsatellites used in this study.

STR	Allele	AF females	AF males
LEX003	**194**	0.1413	0.2137
	198	0.0326	0.0342
	200	0.0072	0.0427
	202	0.0000	0.0085
	204	0.0254	0.0256
	206	0.3225	0.2479
	208	0.1014	0.0940
	210	0.2065	0.1709
	212	0.0290	0.0171
	214	0.1341	0.1368
	216	0.0000	0.0085
LEX026	**300**	0.1522	0.1849
	302	0.0000	0.0252
	304	0.0000	0.0084
	306	0.0833	0.0840
	308	0.0543	0.0336
	310	0.0000	0.0084
	312	0.3152	0.2857
	314	0.3913	0.3697
	318	0.0036	0.0000
TKY270	**156**	0.1728	0.2783
	164	0.0074	0.0348
	166	0.1176	0.0609
	168	0.4449	0.3826
	170	0.2537	0.2261
	172	0.0037	0.0174
TKY38	**105**	0.0906	0.1441
	107	0.0725	0.1695
	109	0.3949	0.3432
	127	0.0036	0.0000
	129	0.3877	0.3008
	131	0.0217	0.0085
	139	0.0072	0.0000
	141	0.0217	0.0339
UCDEQ502	**162**	0.0531	0.0787
	164	0.4735	0.4607
	166	0.0398	0.0112
	168	0.2434	0.3090
	170	0.0177	0.0000
	172	0.0000	0.0112
	174	0.1726	0.1180
	176	0.0000	0.0112

AF females and AF males: allelic frequencies observed in females and males respectively. Frequencies per allele are characterized as fraction of the total determinations segregated by sex of the individuals.

Screening capability

Specificity in the study sample was 99.82%, estimated on the probability to find the five STR-markers in homozygosity in females with normal karyotype (other possibilities are covered by the different result combinations). Likewise, the sensitivity of the diagnostic tool was 100%, since the possibility to obtain a diallelic result in animals with only one ECAX is null.

Validation of the methodology in chromosomally abnormal individuals

The molecular markers used in this study allowed to determine the presence or absence of ECAX and ECAY and the number of chromosomes with a different genetic background (cell lines) through the number of alleles detected. In the same reaction we also determined the presence of the *SRY* gene, which has a crucial role in the case of sex reversal horses. Based on the combinations of the results of those seven markers we detected, with a high confidence, most of the 90% of the sex chromosomal pathologies previously associated with reproductive failures in horses.

Table 4: Molecular profiles of *ECAX*, *ECAY* STR markers and *SRY* gene obtained using the methodology described in this study associated to the most important sex chromosomal abnormalities described in horses.

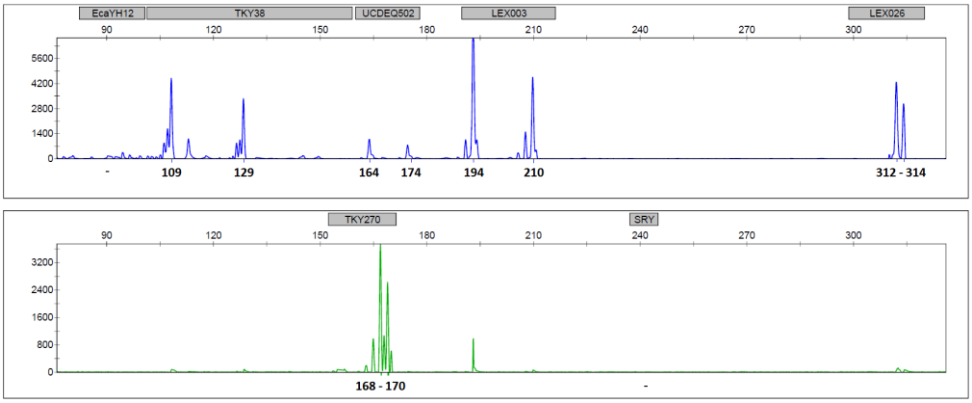
Phenotype	**Molecular Genotype**			**Karyotype**	**Diagnostic**
	ECAX STR´s profile	**ECAY Marker**			
		ECAYH12	***SRY***		
Male	One allele per marker	+	+	64 XY	Normal male
Female	At least one marker in heterozygosis	-	-	64 XX	Normal female
Female	One allele per marker	-	-	63 X0	Turner syndrome*
Male	At least one marker with 2 or 3 alleles	+	+	XX/XY*	Male chimerism**
Female	At least one marker with 2 or 3 alleles	+	+	XX/XY*	Female chimerism**
Male	At least one marker in heterozygosis	-	+	64 XX	Male *SRY* positive DSD***
Male	At least one marker in heterozygosis	-	-	64 XX	Male *SRY* negative DSD
Female	One allele per marker	+	+	64 XY	Female *SRY* positive DSD
Female	One allele per marker	+	-	64 XY	Female *SRY* negative DSD

Diagnostics are performed as the combination of the phenotype and molecular genotype of the individuals. Karyotype stated is the associated to every different syndrome. *Results on Turner´s syndrome have a specify of 99,82%. **Proportion of cell lines may change among individuals but it not affects the results. Different results obtained from the blood and hair DNA samples demonstrate blood chimerism. Similar results demonstrate true chimerism. *** 64,XX *SRY* + DSD was not reported yet in horses but it can be detected with this methodology.

All the individuals studied using the molecular methodology proposed in this report were diagnosed correctly. Table 4 shows karyotypes and STR-profiles in animals used as positive controls. Mares with normal karyotype (64,XX) showed non-amplified ECAY markers and at least one ECAX marker in heterozygosity (two alleles) (Supplementary figure 1, SF1). Males with normal karyotype (64,XY)

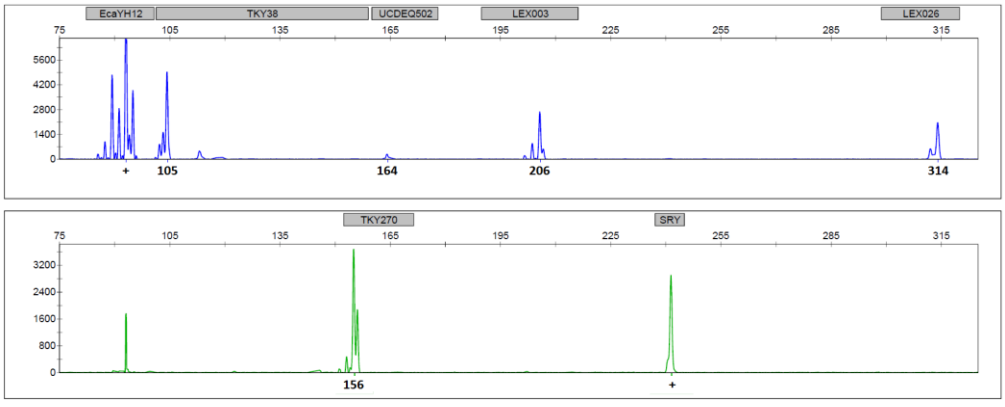
showed amplification in ECAY markers and only one allele peak in ECAX markers (SF2). Individuals affected by sex reversal syndrome showed karyotypes and the STR-profile opposite to their phenotype, being also discriminated by the presence or absence of *SRY* (SF3, SF4 and SF5). Animals affected by Turner´s Syndrome (63,X) showed monoallelic results in all ECAX markers and no amplification in ECAY markers (SF6). Finally, results from chimeric animals were characterized by a positive amplification in ECAY markers and a multiallelic profile (2 or 3 alleles) in at least one of the ECAX markers (SF7). Differences between results from DNA obtained from blood and hair samples indicated blood chimerism. Blood DNA showed the STR chimeric profile and hair DNA showed a normal STR profile according to the sex of the individual (male of female). On the other hand, if the STR chimeric profile is detected in the blood and hair DNA samples the individual is diagnosed as a true (full body) chimerism.

Supplementary figure 1: Electropherogram profile of a normal female (64,XX)



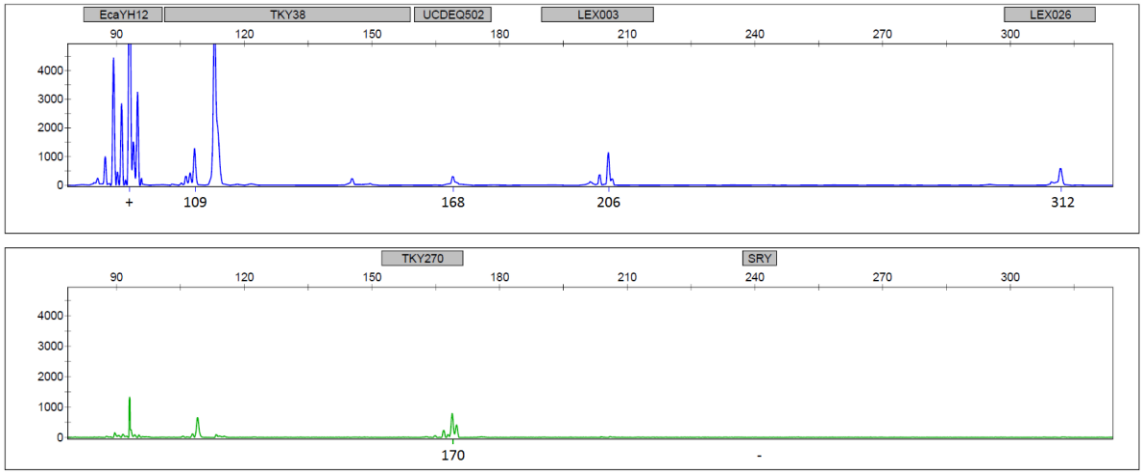
Absence of amplification on ECAY (*ECAYH12* and *SRY*) and diallellic results in more than one ECAX STR (*TKY38*, *UCDEQ502*, *LEX003* and *LEX026* in this case)

Supplementary figure 2: Electropherogram profile of a normal male



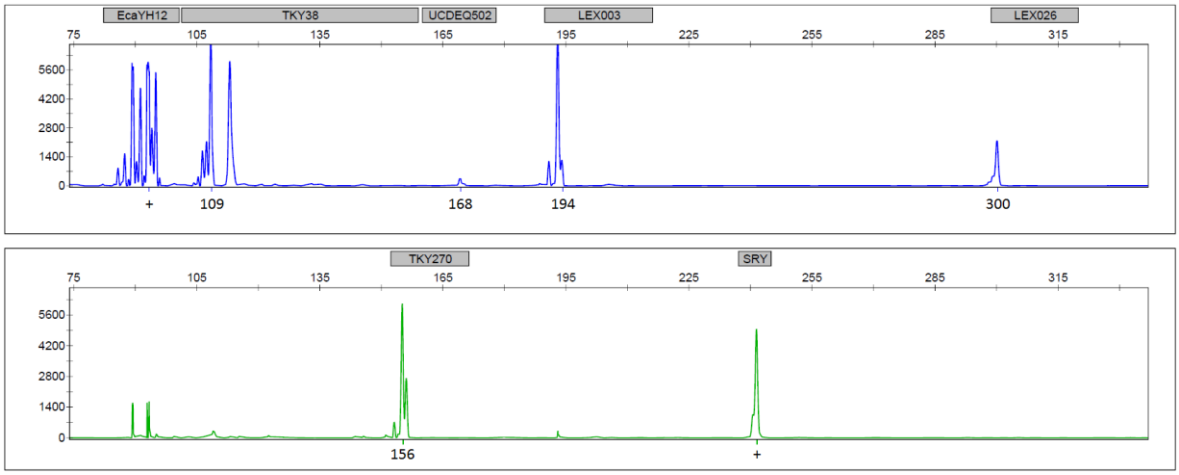
Amplification on both ECAY markers (ECAYH12 and SRY) and monoallellic results in all the ECAX STRs

Supplementary figure 3: Electropherogram profile of a 64 XY, *SRY*-negative DSD mare (sex reversal mare)



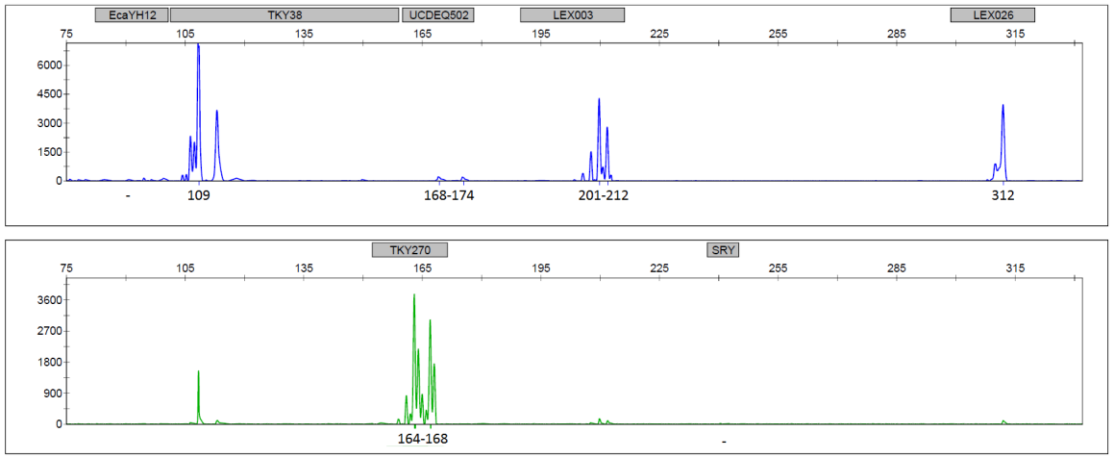
Positive amplification on constitutive ECAY marker (ECAYH12) and negative in SRY fragment, with monoallellic results in all of the ECAX STR´s.

Supplementary figure 4: Electropherogram profile of a 64 XY, *SRY*-positive DSD mare (sex reversal mare)



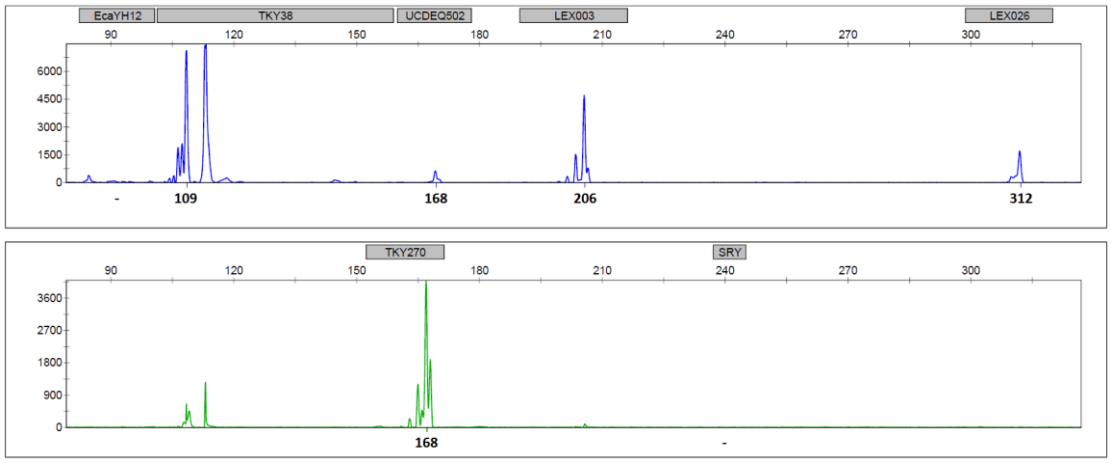
Positive amplification on ECAY´s marker (*ECAYH12* and *SRY*) with monoallellic results in all of the ECAX STR´s, compatible with male profile. The animal was diagnosed as sex reversal since it was phenotypically a female.

Supplementary figure 5: Electropherogram profile of a 64 XX, *SRY*-negative DSD male (sex reversal horse)



Negative amplification on ECAY´s markers (*ECAYH12* and *SRY*) with diallellic results in all of the ECAX STR´s, compatible with female profile. The animal was diagnosed as sex reversal since it was phenotypically a male.

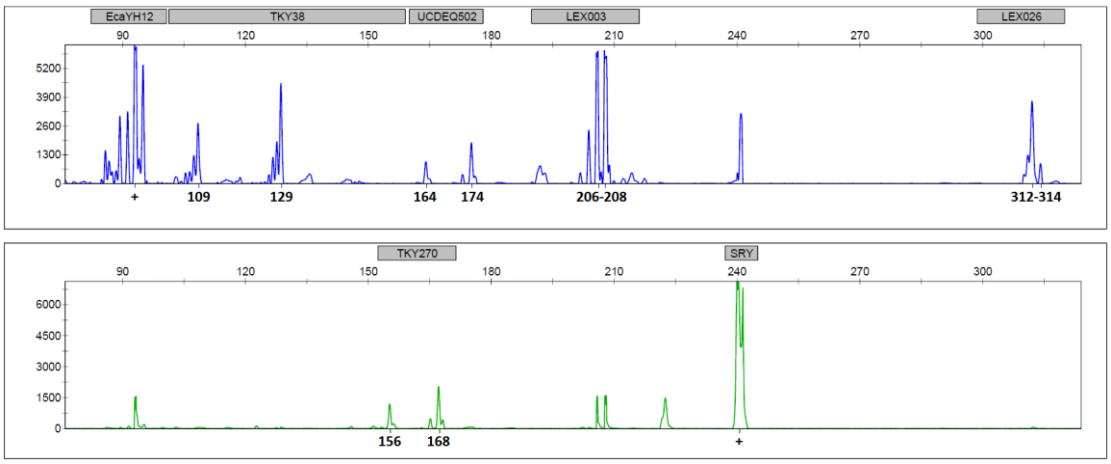
Supplementary figure 6: Electropherogram profile of a Turner´s profile (2n=63,X)



No amplification on both ECAY markers (ECAYH12 and SRY) and monoallellic results in all of the ECAX STRs.

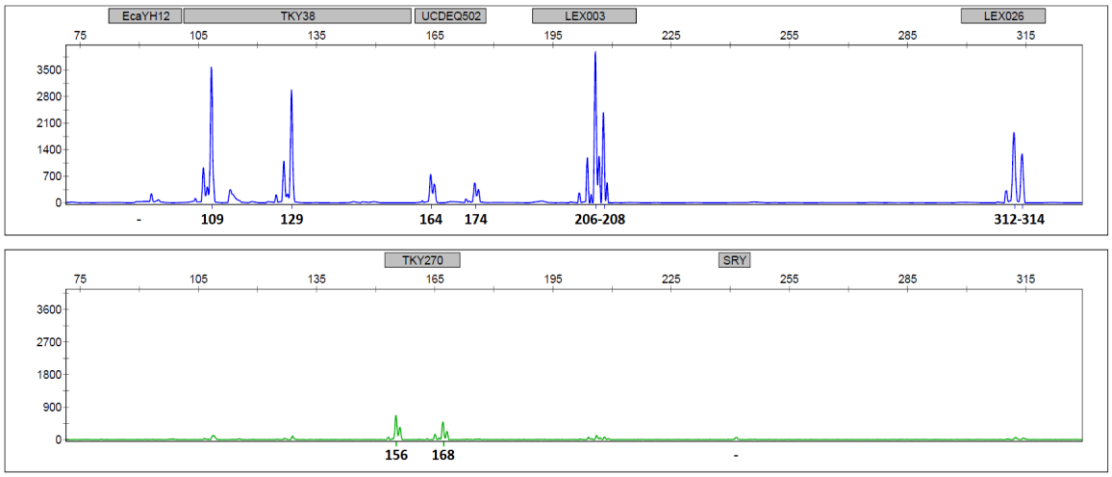
Supplementary figure 7: Electropherogram profiles of blood and hair DNA of an individual with a blood chimerism.

Blood DNA profile



Amplification on both ECAY markers (*ECAYH12* and *SRY*) and diallellic results in at least one ECAX STR (*TKY38*, *UCDEQ502*, *LEX026* and *TKY270* in this case)

Hair DNA profile



No amplification on both ECAY markers (*ECAYH12* and *SRY*) and diallellic results at least one of the ECAX STRs, in agreement with a female profile (All the markers in this case). Differences between blood and hair DNA confirm the existence of a blood chimerism. Hair DNA results were in agreement with phenotype of the individual (a mare).

Discussion

Among the major syndromes associated with unexplained infertility in horses, Turner´s (63,X) is probably the most reported (Lear & McGee 2012). Our results showed that this abnormality could be diagnosed by using STR markers with high confidence. It is noteworthy that it cannot be detected using the molecular markers routinely employed in domestic animals, such as amelogenin (Hasegawa *et al.* 2000) or zinc finger protein genes (Han *et al.* 2010), since there is no discordance between genotypic and phenotypic sex. Therefore, the number of undiagnosed cases could be higher and the prevalence underestimated. In this pathology, the diagnostic confidence obtained by using our approach was higher than 98%, since we saturated the ECAX with several highly variable markers which allows the detection of the number of chromosomes present.

The second most important chromosomal pathology in horses is the sex reversal syndrome. According to Villagómez *et al.* (2009), only three of them have already been reported, with the exception of 64,XX *SRY*-positive DSD individuals. This difference with other species, such as humans (Ergun-Longmire *et al.* 2005), is most likely due to the fact that *SRY* gene is located in a specific region far from the pseudo-autosomal boundary region (PAB), making the gene exchange between ECAY and ECAX unlikely (Raudsepp et al. 2010). It has been demonstrated that 64,XY *SRY*-negative DSD (disorder in the sex development) females are the most common presentation of sex reversal horses (Lear & McGee 2012). Its clinical presentation is characterized by the same phenotypic presentation as that in Turner´s mares, with the consequent high percentage of undiagnosed cases until adulthood (Anaya *et al.* 2014). Our methodology also provides a simple and reliable alternative to detect the four possible presentations of the sex reversal syndrome (according to their chromosomal complements and presence or absence of *SRY*) based on a simple blood sample, which could to decrease the number of undiagnosed horses. However, this methodology cannot discriminate the cause of that abnormality, which can range from point mutations (Révay *et al.* 2012) to small or even large chromosomal deletions (Raudsepp *et al.* 2010).

Another advantage of the methodology developed here is its possibility to discriminate between true and blood chimerisms. Currently, the effect of male-female blood chimerism on horse fertility is still under discussion. Previous reports suggest that blood chimeras are normal (Bouters & Vandeplassche 1972; Juras *et al.* 2010; Demyda-Peyras *et al.* 2013), even when one of the lines has been diagnosed as aberrant (Demyda-Peyrás *et al.* 2014). However, Bugno *et al.* (1999) reported idiopathic subfertility in a mare that delivered for the first time after four reproductive seasons. Considering that the twining rate in horses was estimated at approximately 3.5% of gestations (Wolc *et al.* 2006), and that about 15% of those pregnancies produced at least a live foal (Miller & Woods 1988), we speculate that the percentage of blood chimeric individuals among horses should be much higher than the reported cases. However, since most of the studies agree with the lack of reproductive symptoms in chimeric mares, it is highly possible that most of those animals remain undiagnosed. On the contrary, true chimerism is a different condition characterized by the fusion of two zygotes or early embryos in a single individual. It has already been reported in horses (Dunn et al. 1981) and other domestic species (Batista *et al.* 2000), and there is agreement about the fact that true chimerism is associated with major reproductive abnormalities in animals (Padula 2005). The ability of the methodology described in the present study to differentiate between these two presentations would allow veterinary practitioners and breeders to manage more appropriately the individuals affected, since true chimeric individual are sterile. In the other hand, the use of molecular screening could allow to determine the real prevalence of blood chimerism in horses.

All diagnostic tests are characterized by their sensitivity and specificity (Van Der Schouw *et al.* 1995). Screening tests, for instance, are used to quickly determine the presence of some disease in a population, and must therefore be highly sensitive to avoid by all means the detection of false negatives. In molecular tests based on the combination of individual results of different STRs, sensitivity and specificity are directly related to the number of markers employed and their variability (Garvin *et al.* 1998). We demonstrated that the markers evaluated in our study were highly polymorphic (elevated PIC) with a high number of possible allele combinations, covering a large number of homozygous and heterozygous genotypes. With this information, the probability of a "false female positive" result (a mare with a normal

karyotype diagnosed as Turner´s syndrome) was 0.0017, estimating that only one out of every 588 mares would be erroneously identified as affected by this condition (false positive). In this study, validated in 30 animals karyotyped, the accuracy of the diagnostic tool was 100%.On the opposite, the possibility of a false negative result is ruled out since there is no possibility of getting more than two alleles in non-chimeric animals (only one or two ECAX and therefore a maximum of one or two alleles) or to obtain an heterozygous result in a marker in animals with only one ECAX (63,X or sex reverse animals). While sensitivity remains fixed across horse populations, specificity could vary as a function of the allele frequencies of the STRs used in the different breeds. In this sense, the PICs of several microsatellite markers previously reported in different Japanese (Tozaki *et al.* 2003), European (Achmann *et al.* 2004) and Spanish (Cañon *et al.* 2000) horse breeds were similar to that obtained in our study. This fact strongly suggests that this methodology will remains highly sensitivity despite the breed analyzed.

The only existing population study determining the percentage of chromosomal abnormalities in horses reported a prevalence close to 2% in certain polish autochthonous breeds (Bugno *et al.* 2007). Our results did not show any sex-related chromosomal abnormalities in the 261 newly-analyzed animals, suggesting that their prevalence in PRE horses is lower. Our DNA samples were collected from animals belonging to the PRE Stud book but specifically selected to minimize the coancestry among individuals, maximizing the genetic differences. With these criteria, the allele variability present in the population was determined more properly. However, all the subjects analyzed showed a normal phenotype, probably because infertile animals are usually excluded from the official registers by breeders, since they could be considered as bad publicity for their herds. Yet, the lack of positive results could also be due to the reduced number of individuals screened. Due to that, a solid estimation of the real prevalence of sex chromosomal abnormalities in PRE cannot be performed unless that a higher number of randomly selected animals is analyzed.

In conclusion, we demonstrated that the use of molecular markers could be an interesting option to detect and screen the most important sex chromosomal abnormalities in the horse in a fast, cheap and robust way. In addition, we estimated the sensitivity and specificity of this methodology in a given population of

European horses, obtaining very reliable results (higher than 99.5% in both cases). However, to obtain a more accurate idea of the prevalence of sex chromosomal abnormalities in these breeds, an increased number of individuals should be analyzed. Overall, the use of this strategy as a screening tool integrated to official breed programs could systematically detect, in an early and efficient way, the presence of reproductively impaired individual, saving time and money of veterinarians and breeders.

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References

Achmann, R., I. Curik, P. Dovc, T. Kavar, I. Bodo, F. Habe, E. Marti, J. Sölkner and G. Brem (2004). "Microsatellite diversity, population subdivision and gene flow in the Lipizzan horse." Animal Genetics **35**(4): 285-292.

Anaya, G., M. Moreno-Millán, M. Bugno-Poniewierska, K. Pawlina, A. Membrillo, A. Molina and S. Demyda-Peyrás (2014). "Sex reversal syndrome in the horse: Four new cases of feminization in individuals carrying a 64,XY SRY negative chromosomal complement." Animal Reproduction Science **151**(1-2): 22-27.

Bannasch, D., C. Rinaldo, L. Millon, K. Latson, T. Spangler, S. Hubberty, L. Galuppo and L. Lowenstine (2007). "SRY negative 64,XX intersex phenotype in an American saddlebred horse." Veterinary Journal **173**(2): 437-439.

Batista, M., F. González, F. Cabrera, E. Palomino, E. Castellano, P. Calero and A. Gracia (2000). "True hermaphroditism in a horned goat with 60XX/60XY chimerism." Canadian Veterinary Journal **41**(7): 562-564.

Belkhir, K., P. Borsa, L. Chikhi, N. Raufaste and F. Bonhomme (1996-2004). GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations, Laboratoire Génome, Populations, Interactions, CNRS UMR 5171.

Berta, P., J. R. Hawkins, A. H. Sinclair, A. Taylor, B. L. Griffiths, P. N. Goodfellow and M. Fellous (1990). "Genetic evidence equating SRY and the testis-determining factor." Nature **348**(6300): 448-450.

Bouters, R. and M. Vandeplassche (1972). "Twin gestation in the mare: the incidence of placental vascular anastomoses and their influence on the reproductive performance of heterosexual equine twins." Journal of Reproduction and Fertility **29**(1): 149.

Bugno, M., E. Słota and M. Kościelny (2007). "Karyotype evaluation among young horse populations in Poland." Schweizer Archiv fur Tierheilkunde **149**(5): 227-232.

Bugno, M., E. Słota, M. Tischner and A. Kozubska-Sobocińska (1999). "A case of 64,XX/64,XY leukocytic chimerism in a fertile mare of the Wielkopolska breed." Ann. Anim. Sci. **26**: 9-16.

Cañon, J., M. L. Checa, C. Carleos, J. L. Vega-Pla, M. Vallejo and S. Dunner (2000). "The genetic structure of Spanish Celtic horse breeds inferred from microsatellite data." Animal Genetics **31**(1): 39-48.

Coogle, L., E. Bailey, R. Reid and M. Russ (1996). "Equine dinucleotide repeat polymorphisms at loci LEX002, -003, -004, -005, -007, -008, -009, -010, -011, -013 and -014." Animal genetics **27**(2): 126-127.

Coogle, L., R. Reid and E. Bailey (1996). "Equine dinucleotide repeat loci from LEX025 to LEX033." Animal Genetics **27**(4): 289-290.

Demyda-Peyrás, S., G. Anaya, M. Bugno-Poniewierska, K. Pawlina, A. Membrillo, M. Valera and M. Moreno-Millán (2014). "The use of a novel combination of diagnostic molecular and cytogenetic approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism." Theriogenology **81**(8): 1116-1122.

Demyda-Peyras, S., M. Bugno-Poniewierska, K. Pawlina, G. Anaya and M. Moreno-Millán (2013). "The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: A Case of sex chromosome chimerism in a Spanish Purebred colt." Cytogenetic and Genome Research **141**: 277-283.

Dunn, H. O., D. Smiley, J. R. Duncan and K. McEntee (1981). "Two equine true hermaphrodites with 64,XX/64,XY and 63,XO/64,XY chimerism." The Cornell veterinarian **71**(2): 123-135.

Eggleston-Stott, M. L., A. DelValle, M. Bautista, S. Dileanis, E. Wictum and A. T. Bowling (1997). "Nine equine dinucleotide repeats at microsatellite loci UCDEQ136, UCDEQ405, UCDEQ412, UCDEQ425, UCDEQ437, UCDEQ467, UCDEQ487, UCDEQ502 and UCDEQ505." Animal Genetics **28**(5): 370-371.

Ergun-Longmire, B., G. Vinci, L. Alonso, S. Matthew, S. Tansil, K. Lin-Su, K. McElreavey and M. I. New (2005). "Clinical, hormonal and cytogenetic evaluation of 46,XX males and review of the literature." Journal of Pediatric Endocrinology and Metabolism **18**(8): 739-748.

Fernández, M. E., D. E. Goszczynski, J. P. Lirón, E. E. Villegas-Castagnasso, M. H. Carino, M. V. Ripoli, A. Rogberg-Muñoz, D. M. Posik, P. Peral-García and G. Giovambattista (2013). "Comparison of the effectiveness of microsatellites and SNP panels for genetic identification, traceability and assessment of parentage in an inbred Angus herd." Genetics and Molecular Biology **36**(2): 185-191.

Ford, C. E., K. W. Jones, P. E. Polani, J. C. De Almeida and J. H. Briggs (1959). "A sex-chromosome anomaly in a case of gonadal dysgenesis (Turner's syndrome)." The Lancet **273**(7075): 711-713.

Garvin, A. M., W. Holzgreve and S. Hahn (1998). "Highly accurate analysis of heterozygous loci by single cell PCR." Nucleic Acids Research **26**(15): 3468-3472.

Han, S. H., B. C. Yang, M. S. Ko, H. S. Oh and S. S. Lee (2010). "Length difference between equine ZFX and ZFY genes and its application for molecular sex determination." Journal of Assisted Reproduction and Genetics **27**(12): 725-728.

Hasegawa, T., F. Sato, N. Ishida, Y. Fukushima and H. Mukoyama (2000). "Sex Determination by Simultaneous Amplification of Equine SRY and Amelogenin Genes." Journal of Veterinary Medical Science **62**(10): 1109-1110.

Hirota, K., T. Tozaki, S. Mashima and N. Miura (2001). "Cytogenetic assignment and genetic characterization of the horse microsatellites, TKY4-18, TKY20, TKY22-24, TKY30-41 derived from a cosmid library." Animal Genetics **32**(3): 160-162.

Juras, R., T. Raudsepp, P. J. Das, E. Conant and E. G. Cothran (2010). "XX/XY Blood Lymphocyte Chimerism in Heterosexual Dizygotic Twins from an American Bashkir Curly Horse. Case Report." Journal of Equine Veterinary Science **30**(10): 575-580.

Kakoi, H., K. Hirota, H. Gawahara, M. Kurosawa and M. Kuwajima (2005). "Genetic diagnosis of sex chromosome aberrations in horses based on parentage test by microsatellite DNA and analysis of X- and Y-linked markers." Equine Veterinary Journal **37**(2): 143-147.

Kakoi, H., T. Tozaki, K. Hirota, S. Mashima, M. Kurosawa and N. Miura (2000). "Ten equine microsatellite loci: TKY25, TKY26, TKY27, TKY28, TKY29, TKY267, TKY268, TKY269, TKY270 and TKY271." Animal Genetics **31**(1): 68-69.

Kalinowski, S. T., M. L. Taper and T. C. Marshall (2007). "Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment." Molecular Ecology **16**(5): 1099-1106.

Kent, M. G., R. N. Shoffner, A. Hunter, K. O. Elliston, W. Schroder, E. Tolley and S. S. Wachtel (1988). "XY Sex reversal syndrome in the mare: Clinical and behavioral studies, H-Y phenotype." Human Genetics **79**(4): 321-328.

Lear, T. L. and E. Bailey (2008). "Equine clinical cytogenetics: The past and future." Cytogenetic and Genome Research **120**(1-2): 42-49.

Lear, T. L. and R. B. McGee (2012). "Disorders of sexual development in the domestic horse, Equus caballus." Sexual Development **6**(1-3): 61-71.

Miller, A. and G. L. Woods (1988). "Diagnosis and correction of twin pregnancy in the mare." The Veterinary clinics of North America. Equine practice **4**(2): 215-220.

Padula, A. M. (2005). "The freemartin syndrome: An update." Animal Reproduction Science **87**(1-2): 93-109.

Pailhoux, E., E. P. Cribiu, P. Parma and C. Cotinot (1995). "Molecular analysis of an XY mare with gonadal dysgenesis." Hereditas **122**(2): 109-112.

Power, M. M. (1987). The Chromosomes of the Horse: Karyotype Definition, Clinical Applications and Comparative Interspecies Studies, University College Dublin.

Raudsepp, T., K. Durkin, T. L. Lear, P. J. Das, F. Avila, P. Kachroo and B. P. Chowdhary (2010). "Molecular heterogeneity of XY sex reversal in horses." Anim Genet **41 Suppl 2**(SUPPL. 2): 41-52.

Révay, T., D. A. F. Villagómez, D. Brewer, T. Chenier and W. A. King (2012). "GTG mutation in the start codon of the androgen receptor gene in a family of horses with 64,XY disorder of sex development." Sexual Development **6**(1-3): 108-116.

Richer, C. L., M. M. Power, L. R. Klunder, R. A. McFeely and M. G. Kent (1990). "Standard karyotype of the domestic horse (Equus caballus). Committee for standardized karyotype of Equus caballus. The Second International Conference for Standardization of Domestic Animal Karyotypes, INRA, Jouy-en Josas, France, 22nd-26th May 1989." Hereditas **112**(3): 289-293.

Rodero-Serrano, E., S. Demyda-Peyrás, A. González-Martinez, A. Rodero-Franganillo and M. Moreno-Millán (2013). "The rob(1;29) chromosome translocation in endangered Andalusian cattle breeds." Livestock Science **158**(1-3): 32-39.

Sánchez-Guerrero, M. J., A. Molina, M. D. Gómez, F. Peña and M. Valera (2016). "Relationship between morphology and performance: Signature of mass-selection in Pura Raza Español horse." Livestock Science **185**: 148-155.

Sumner, A. T. (1972). "A simple technique for demonstrating centromeric heterochromatin." Experimental Cell Research **75**(1): 304-306.

Tozaki, T., N. Takezaki, T. Hasegawa, N. Ishida, M. Kurosawa, M. Tomita, N. Saitou and H. Mukoyama (2003). "Microsatellite Variation in Japanese and Asian Horses and Their Phylogenetic Relationship Using a European Horse Outgroup." Journal of Heredity **94**(5): 374-380.

Van Der Schouw, Y. T., A. L. M. Verbeek and S. H. J. Ruijs (1995). "Guidelines for the assessment of new diagnostic tests." Investigative Radiology **30**(6): 334-340.

Villagómez, D. A. F., T. L. Lear, T. Chenier, S. Lee, R. B. McGee, J. Cahill, R. A. Foster, E. Reyes, E. St John and W. A. King (2011). "Equine disorders of sexual development in 17 mares including XX, SRY-negative, XY, SRY-negative and XY, SRY-positive genotypes." Sexual Development **5**(1): 16-25.

Villagómez, D. A. F., P. Parma, O. Radi, G. Di Meo, A. Pinton, L. Iannuzzi and W. A. King (2009). "Classical and molecular cytogenetics of disorders of sex development in domestic animals." Cytogenetic and Genome Research **126**(1-2): 110-131.

Wallner, B., F. Piumi, G. Brem, M. Müller and R. Achmann (2004). "Isolation of Y Chromosome-specific Microsatellites in the Horse and Cross-species Amplification in the Genus Equus." Journal of Heredity **95**(2): 158-164.

Wolc, A., A. Bresińska and T. Szwaczkowski (2006). "Genetic and permanent environmental variability of twinning in Thoroughbred horses estimated via three threshold models." Journal of Animal Breeding and Genetics **123**(3): 186-190.

CAPÍTULO V

Capítulo V:Blood chimerism in the horse is reproductively innocuous? a 28 cases report.

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Abstract

Chromosomal abnormalities are known to be a major genetic cause of horse infertility, remaining undiagnosed in a high percentage of the cases. Among them, chimerism has been considered rare in this specie due the lack of viability of twin pregnancies in mares. Due to that, the number of chimeric horses reported is minimal, in comparison with other livestock species. In this study we reported for the first time fifteen Pura Raza Español (PRE) showing blood sex chimerism (64,XX/64,XY), being eight of them twins siblings. Animals showed three and even four different alleles in several loci in the Short Tandem Repeat (STR) DNA parentage tests used by the Spanish Purebred Breeders Association. Results were confirmed using a newly-developed molecular tool which evaluates specifically the presence of different cell lines in the sex chromosome pair. Differences observed between blood and hair DNA determined that all the cases were blood chimerism. We also determined for the first time the presence of two twins siblings which did not show any sign of chimerism. All the animals were characterized as reproductively normal, in the morphology, and behavior and most of them produced healthy offspring. Our comprehensive study strongly suggests than the blood chimerism in horses is reproductively innocuous, being all the blood chimeric horses fertile. Due to that, we suggest that the number of individuals carrying this chromosomal abnormality could be largely underestimated.

Introduction

Equine cytogenetic is poorly developed around the world, probably due to the fact that the horse karyotype is particularly complex and difficult to analyze (Lear & Bailey 2008). Another reason is that probes for in situ hybridization (whole chromosome or BAC derived probes) are only available in

research laboratories (Bugno et al. 2009; Raudsepp et al. 2010). In the same way, the use of comparative genomic hybridization (CGH), which was recently developed in this species (Bugno-Poniewierska et al. 2016), is not available to evaluate chromosome abnormalities in the sexual pair due to the lack of ECAY reference sequence. Nowadays, and despite horses showed an increased prevalence of chromosomal abnormalities compared with any other domestic animal, there are no commercial screening tests available yet.

Chromosomal abnormalities are one of the most important genetic pathologies associated with equine infertility. Some of them are easy to recognize since they are associated with major morphological abnormalities (De Lorenzi et al. 2010). However, a high percentage of these pathologies remains silent until puberty, when morphological or physiological abnormalities become visible (Anaya et al. 2014). Among them, chromosomal sexual chimerism (64,XX/64,XY) is rarely reported in horses. This pathology could be produced either by the exchange of blood precursor cells trough placenta circulation between dizygotic twins (blood chimerism) (Vandeplassche et al. 1970), or by the fusion of two embryos at the very early stages of development (true chimerism, Dunn et al. (1981). In horses, twinning is a rare condition since the viability of fetuses and even the mare are questionable and, therefore, this condition is treated as a pathology (Tan & Krekeler 2014). Thus, most studies assessing sexual chimerism in live horses were single case reports. To our knowledge, there is only one case analyzing cytogenetically twin foals (Juras et al. 2010), without finding any morphological abnormality. Conversely, the most comprehensive study assessing chimerism in horse pregnancies, performed 45 years ago, showed that only 11 out of 25 fetuses derived from twin gestations were chimeric.

Short tandem repeat markers (STR) were previously used to determine chromosomal abnormalities in horses, including cases of blood chimerism (Kakoi et al. 2005; Juras et al. 2010; Demyda-Peyras et al. 2013). We recently validated a methodology which can determine the presence of such abnormalities in a cheap and easy way based on the analysis of a panel of molecular markers located in the ECAY and ECAX (Anaya et al. 2016). This technique was partially used in a previous case of blood chimerism (Demyda-Peyrás et al. 2014) and could increase the detection rate since it can be easily used in a large

number of individuals. Therefore, the number of individuals analyzed could be largely increased with the implementation of this technique in the breeding schemes currently developed.

To our knowledge, this is the most comprehensive study assessing blood chimerism in live horses and their reproductive implications. Individuals were diagnosed using a newly developed molecular methodology. Additionally, the reproductive and morphological data available were used to determine the fertility of the individuals, assessing the effect of blood chimerism in horses.

Material and methods

Animals and reproductive assessment

Fifteen animals (6 individuals showing a male phenotype and 9 individuals showing a female phenotype) belonging to the Asociación Nacional de Criadores de Caballo Pura Raza Español (ANCCE) were analyzed (Table 1). Animals ranged from 2 months to 16 years of age when the analysis was performed. All the animals were initially inspected after birth by breeders to assess the absence of large external morphological defects. Later, at the age of 1 year, the individuals were re-inspected by the official veterinary services of the breeders´ association to determine if they complied are with the breed standards and to exclude the presence of disqualifying defects in their morphology. During this assessment, blood samples are collected to filiation test. Finally, after the individuals reached the age of 3, those intended to become breeders were re-inspected by official veterinaries to obtain the "basic reproductive approval". This examination included visual inspections of the internal and external reproductive organs and the sexual behavior of the candidates. In mares, the occurrence of reproductive cycles and ovulation is also determined.

Samples

Animals were sampled as a part of the ANCCE breeding program. Blood DNA was obtained by jugular venopuncture Tri-sodium EDTA BD VacutainersTM and the Blood Genomic DNA Extraction Kit. Hair follicle DNA was obtained using the Tissue Genomic DNA Purification Kit (Canvax, Cordoba, Spain)

according to the manufacturers´ protocols. In some cases, only blood DNA was obtained directly from the ANCCEE, since the present localization and status of the individual are unknown.

Genotyping

Animals were genotyped according the International Society of Animal Genetic (ISAG) guidelines with a panel of 17 STR markers (Supplementary table 1). Determinations were performed using the StockMarks® for Horses 17-Plex Genotyping Kit (Thermofisher Scientific, Madrid, Spain). Additionally, genetic sex was also determined in all the individuals using *AMX/Y* gene, according to Hasegawa *et al.* (2000).

Supplementary table 1: Results of Sort Tandem Repeats (STR) using QF-PCR of DNA obtained from blood and hair samples

STR Marker	Size Range (bp)	Position (ECA)
AHT4	140-166	24
AHT5	126-147	8
ASB17	104-116	2
ASB2	237-268	15
ASB23	176-212	3
CA425	224-247	28
HMS1	166-178	15
HMS2	215-236	10
HMS3	146-170	3
HMS6	154-170	4
HMS7	167-187	1
HTG10	83-110	21
HTG4	116-137	9
HTG6	74-103	15
HTG7	114-128	4
LEX003	137-160	X
VHL20	83-102	30

STR markers used in the official paternity diagnostic panel suggested by ISAG. All the markers were determined in a simple reaction according to (Dimsoski 2003)

Individuals were further genotyped using a recently developed sex linked STR-panel (Anaya et al. 2016), including five ECAX (LEX003, LEX0026, TKY38, TKY270, and UCEDQ502) and two extra ECAY-linked (ECAYM2 and SRY) markers. Determinations were resolved in a single PCR reaction according to our protocols with a specific set of labeled primers (Supp Table 2). PCR was performed in a 23 mL reaction mixture containing 20 to 60 ng of genomic DNA,1.5 to 7.5 pmol of each primer pair, 0.33mmol/

L of dNTPs, 2.5mmol/L of MgCl2,1X PCR reaction buffer, and 1.5 U of Horse-Power™ Taq DNA Polymerase (Canvax Biotech, Cordoba, Spain). Samples were initially denatured at 95 ºC for 10 min, followed by 33 cycle of 94 ºC for 30 s, 57 ºC for 1min, and 72 ºC for 30 s, with a final elongation step of 10 min at 72 ºC. Amplification products were then genotyped by capillary sequencing at the SCAI genomics core, University of Cordoba, Spain). Allele allocation was made processing the raw data with the Genotyper 4.0 software package using a LIZ 500 bp internal size standard (Applied Biosystems).

Supplementary Table 2: ECAX and ECAY molecular markers employed in this study.

Name	Marker type	Amplicon Size	Primer sequence (5´to 3´)	Chromosome	Accesion	Reference
LEX003	STR	194-214	ACATCTAACCAGTGCTGAGACT AAGACTAGACCTACAACTAGG	X	AF075607.1	A
LEX026	STR	300-314	TCCAGAGTGAATGGCAAATC AAGAACTAGAACCTACAACTAGG	X	AF075628.1	B
TKY38	STR	105-166	TAAGTATTCTCATAAACGGG GGAATAATAACAGCATCCTC	X	AB048344.1	C
TKY270	STR	154-172	CTGCTTTAGAGAAACAAACT CCATGGTGAGAAAAATGAGA	X	AB048312.1	D
UCDECA502	STR	150-170	AGAGGGCAAAGTCAGAGCTT AGCACCTGATGCTTCTTGTT	X	U67420.1	E
ECA.YM2	STR	118	TGGTTCAGATGGTGTATTTTGTT TTTGCAGCCAGTACCTACCTT	Y	BV005726	F
SRY	Gene	249	TACCACCCTCCTCTTCAACG ATCACGAGACCACACCATGA	Y	AB004572	G
ZFX	Gene	604	AAATCAAAACCTTCATGCCAAT	X	DQ179230 (ZFX)	G
ZFY		553	TTCCGGTTTTCAATTCCATC	Y	DQ179229 (ZFY)	
KIT exon 21	Gene	785	GCTGTTGGGTTGTAGTTGG CAACCATCCTTTTGGACAG	3 (horse)	106839052	H
AMX	Gene	184	CCAACCCAACACCACCAGCCAAACCTCCCT	X	106840190	I
AMY		164	AGCATAGGGGGCAAGGGCTGCAAGGGGAAT	Y	100034231	

Fragments were amplified using a set of primer pairs previously described by for horses in (Anaya et al. 2014)

Results

Morphological examination and Reproductive parameters

All the individuals showed a normal morphology at the examination (Table 1). Only one mare obtained the reproductive endorsement from ANCCE, producing one healthy offspring. One of the meaning stallion was castrated and the other was reported as diseased without being used as breeders. Three mares were evaluated as breeders, producing 4, 1 and 1 offspring respectively. Veterinary reports showed no reproductive or sex behavioral abnormalities. The three remaining mares were discarded as breeders by

decision of the owners. Finally, all the twin sisters were only evaluated in their external morphology since they were to young at the age of the analysis.

Parentage genotiping STR´s

Table 2 show the STR-profile of the markers used in parentage test in the 15 cases studied. All the individuals showed a normal profile, according with their phenotypic sex in the hair DNA samples. On the contrary, 13 individuals showed an abnormal pattern in the blood DNA samples, being diagnosed as carriers of a blood chromosomal chimerism. It is noteworthy that one individual (mare 2) showed differences in only one marker, which is statically rare. The second pair of twins (TM2 and TS2) showed identical results on blood markers but they were different in DNA samples. Finally, results obtained from blood DNA of the third pair of twins (TM3 and TS3) were different, demonstrating the absence of chimerism despite the fact that they are derived from a single birth.

ECAX and ECAY SRT´s

Results of sex-linked STR markers agree with the obtained in the parentage panel (Table 3). Due to the lack of hair DNA samples only 7 individuals (one stallion and the six twins) were gentoyped using this methodology. In those animals, hair DNA showed results in agreement with the phenotypic sex. Blood DNA showed biallellism in the ECAX linked markers and the presence of ECAY linked markers in remaining individuals, showing the presence of at least two different ECAX and one ECAY in all of them. Interestingly, S2, M1 and the second pair of twins (TM2 and TS2) showed a triallellism in some ECAX linked markers. In the case of the third pair of twin (TM3 and TS3), the results confirmed that blood chimerism was absent in both individuals. Amelogenine results showed the presence of AMX and AMY (linked to ECAX and ECAY respectively) in the blood of all the individuals but only the AMX in the hair of the female twins.

Table 2: Results of Short Tandem Repeats (STR) markers used in parentage test determine from two different DNA source in each individual

	Stallions						Mares								Twins							
	S1		S2		S3		M1		M2		M3		M4		M5		M6		Mare 1		Stallion 1		Mare 2		Stallion 2		Mare 3		Stallion 3		
STR / DNA	B	H	B	H	B	H	B	H	B	H	B	H	B	H	B	H	B	H	B	H	B	H	B	H	B	H	B	H	B	H	ECA
AHT4	HJ	HJ	HJ	HJ	**HJM**	JM	HJK	KK	HH	HH	HJO	HO	**HJO**	JO	**JKO**	KO	IO	IO	JO	-	JO	-	**JK**	K	**JK**	J	JO	JO	HK	HK	24
AHT5	**KLN**	LN	**KNO**		**KMN**	KN	NN	NN	NN	NN	**KMN**	KM	KN	NN	KL	KL	**KLMO**	LO	JK	-	JK	-	KL	KL	KL	KL	KN	KN	K	KK	8
ASB17	**HMR**	HR	KR	KR	**FHKM**	HM	HM	HM	HM	HM	FM	FM	OR	OR	FH	FH	HK	HK	HR	-	HR	-	NR	NR	NR	NR	OR	OR	OR	OR	2
ASB2	KN	KN	**KMN**	KM	**KMQ**	QQ	**IMP**	MP	NQ	NQ	QR	QR	KK	KK	MM	-	IQ	IQ	**MNQ**	-	**MNQ**	-	NQ	NQ	NQ	NQ	MN	MN	KM	KM	15
ASB23	U	U	JL	JL	LL	LL	JKL	JL	**JKS**	KS	KL	KL	JL	JL	JS	JS	**KLS**	LL	JL	-	JL	-	IM	IM	IM	IM	JO	JO	JJ	JJ	3
CA425	NN	NN	MN	MN	JN	JN	LN	LN	MN	MN	MO	MO	NN	NN	GM	GM	**GMN**	MN	JN	-	JN	-	**LN**	NN	LN	LN	MN	MN	MN	MN	28
HMS1	IL	IL	**JMQ**	JQ	JM	MM	JM	JM	M	MM	MQ	MQ	JM	JM	JJ	JJ	**JMQ**	JQ	KK	-	JL	JL	JL	JL	JL	LL	LL	LL	LQ	LQ	15
HMS2	JJ	JJ	**HK**	HH	**HJM**	JM	HR	-	KK	KK	KK	KK	KL	KL	IK	IK	HM	HM	HH	-	HK	HK	HK	HK	HK	HK	KK	KK	KK	KK	10
HMS3	MN	MN	**IOP**	IP	MP	PP	**IMO**	IM	NR	NR	IN	IN	**IMR**	MR	**IMO**	IM	IP	IP	IR	-	IN	IN	IN	IN	IN	IN	RR	RR	RR	RR	3
HMS6	KN	KN	PP	PP	MO	MO	LO	-	KP	KP	OP	OP	KO	KO	OP	OP	**KLP**	KL	LP	-	KL	KL	KL	KL	KL	KL	KM	KM	MM	MM	4
HMS7	LO	LO	NO	NO	NN	NN	NO	NO	KO	KO	OO	OO	NO	NO	NO	NO	NO	NO	KN	-	**MO**	OO	MO	MO	KL	KL	MN	MN	NN	NN	1
HTG10	OS	OS	PP	PP	**OR**	OO	**MQR**	-	OQ	OQ	RR	RR	MO	MO	PR	PR	PP	PP	OO	-	JN	JN	JN	JN	KM	M	-	-	-	-	21
HTG4	KM	KM	KL	KL	**KLM**	KM	K	KK	KM	KM	LN	LN	MM	MM	KM	KM	KK	KK	KK	-	KM	KM	**KM**	M	KN	KN	KM	KM	KM	KM	9
HTG6	JO	JO	GJ	GJ	**JO**	OO	**GOP**	OP	JO	JO	**JO**	OO	**HO**	OO	JJ	JJ	JO	JO	GO	-	**JOP**	JO	**JOP**	JP	JO	JO	KK	KK	JO	JO	15
HTG7	OO	OO	**OR**	OO	NO	NO	OO	OO	NO	NO	MO	MO	KN	KN	**ORS**	OO	KO	KO	OO	-	LO	LO	LO	LO	LO	LO	OO	OO	OO	OO	4
VHL20	LQ	LQ	**LMQF**	LQ	Q	Q	LR	RR	MR	MR	NN	NN	QR	QR	MP	MP	**NR**	RR	MP	-	**MLQ**	LQ	**MLQ**	LM	MQ	MQ	MQ	MQ	LQ	LQ	30

STR: Marker name. DNA source: B: blood extracted DNA. H: hair extracted DNA. ECA: chromosomic location of the marker. Results stated in bold font are different between B and H DNA samples.

Table 3: Results of Short Tandem Repeats (STR) markers used in parentage test determine from two different DNA source in each individual

	Sample	STR / DNA	EcaYH12	SRY	LEX003	LEX026	TKY270	TKY38	UCDEQ502
Stallions	**S1**	**B**	95	249	210	**300/314**	154	129	**166/170**
		H	95	249	210	300	154	129	166
	S2	**B**	95	249	**208/210/214**	**300/306/314**	**164/168**	**105/109/141**	**156/168/170**
		H	-	-	-	-	-	-	-
	S3	**B**	95	249	206/208	312/314	168	129	168/174
		H	-	-	-	-	-	-	-
Mares	**M1**	**B**	**95**	**249**	**208/210/214**	**300/306/314**	**164/168**	**105/109/141**	**156/168/170**
		H	-	-	-	-	-	-	-
	M2	**B**	**95**	**249**	194/210	300/312	156/168	107/109	164/168
		H	-	-	-	-	-	-	-
	M3	**B**	**95**	**249**	206/214	300/314	168	105/109	164/168
		H	-	-	-	-	-	-	-
	M4	**B**	**95**	**249**	210/214	306	168	105/109	162/166
		H	-	-	-	-	-	-	-
	M5	**B**	**95**	**249**	194/206	314	168/170	109	164/174
		H	-	-	-	-	-	-	-
	M6	**B**	**95**	**249**	208	312/314	156/168	109/129	174
		H	-	-	-	-	-	-	-
Twins	**Mare 1**	**B**	**95**	**249**	194	300/308	156/170	129	174
		H	-	-	-	-	-	-	-
	Stallion 1	**B**	95	249	194	**300/308**	**156/170**	129	174
		H	-	-	-	-	-	-	-
	Mare 2	**B**	**95**	**249**	206/214	308/314	**156/168/170**	129	166/176
		H	0	0	206/214	314	168/170	129	166/176
	Stallion 2	**B**	95	249	**206/214**	**308/314**	**156/168/170**	129	**166/176**
		H	95	249	206	308	156	129	166
	Mare 3	**B**	0	0	206/210	312/314	156/168	129	166/170
		H	0	0	206/210	312/314	156/168	129	166/170
	Stallion 3	**B**	95	249	194	312	168	109	170
		H	95	249	194	312	168	109	170
		ECA	Y	Y	X	X	X	X	X

STR: Marker name. DNA source: B: blood extracted DNA. H: hair extracted DNA. ECA: chromosomic location of the marker. Results stated in bold are different between B and H DNA samples.

Discussion

Blood chimerism is a common cause of sterility in several domestic species (Thomsen & Poulsenm 1993; Hinrichs et al. 1999; Brace et al. 2008; Peretti et al. 2008). However, its effect is still controversial in horses. Hereby, we present the most comprehensive study performed in equines, including the characterization of three pairs of live twin foals for the first time. To date, the largest study assessing horse chimerism (25 cases) was performed by Vandeplassche et al. (1970) in twin fetuses, determining that 44% of the individuals analyzed were chimeric. More recently, a few more cases were reported but mainly released as single case reports (Moreno-Millan et al. 1991; Bugno et al. 1999; Bugno et al. 2007; Demyda-Peyras et al. 2013), excepting Juras et al. (2010) who reported blood chimerism in two live foals. However, and despite the high percentage of sex chromosomal abnormalities reported in horses, the phenotypical effect of sex chimerism is still controversial, probably because karyotyping of tissues other than blood is extremely more complicated and thus not performed in practice.

Comparison of results from blood and hair DNA analysis allowed to determine the kind of chimerism, true or blood (Anaya et al. (2016). In our case, all the animals analyzed were diagnosed as blood chimerism. It is well known that true chimeras are characterized by an aberrant conformation in the reproductive organs in several species (Batista et al. 2000; Brace et al. 2008). In horses, both cases were reported: true (McIlwraith et al. 1976; Dunn et al. 1981) and blood (Bugno et al. 2007; Juras et al. 2010; Demyda-Peyras et al. 2013) chimeras. The former resemble the cases described in similar livestock species, in which morphological abnormalities are highly visible. On the contrary, blood chimeras showed a normal phenotype and fertility. In our study, all the mares which obtained the reproductive approval from ANCCE had delivered healthy offspring. On the contrary, although the only male approved as a breeder was not used by the owner´s decision, it showed normal sperm motility and sexual behavior. The lack of abnormal reproductive symptoms or behavior observed in the rest of the animals at reproductive age and the absence of morphological abnormalities in their adulthood strongly suggest that blood chimerism is reproductively neutral in this species. The fact that only one of the males had produced progeny was not attributable to genetics since the remaining two individuals were castrated at

young ages. Finally, the fertility observed in all the individuals agree with the fact that placental anastomoses occurred after the triggering of sex development, which could allow the normal development of the reproductive tract.

It is noteworthy that two previous studies reported reproductive failures associated with blood chimerism in horses. Bugno et al. (1999) reported a normal mare with problems to become pregnant, delivering his first foal at the age of seven. On the contrary, reported two cases of chimerism associated with ovarian hypoplasia, male-like behavior, and infertility, symptoms compatible with Turner´s syndrome and sex reversal mares (Lear & McGee 2012). In both studies, karyotyping was only performed in blood samples, precluding? the differentiation between blood and true chimerism. Therefore, a strong possibility is that both cases could be misdiagnosed as true chimeras, which could better explain the lack of fertility observed. More recently, molecular methods allowed to easily determine between different cases of chimerisms since chromosomal abnormalities could be detected in several tissues (Anaya et al. 2016). Since then, the few studies performed demonstrated that blood chimerism had no reproductive effect on the reported cases (Juras et al. 2010; Demyda-Peyras et al. 2013) even when one of the cell lines detected was aberrant (Demyda-Peyrás et al. 2014).

Twining is one of the most important causes of reproductive complications in horses. This kind of gestation is normally unsuccessful and compromises the mare´s life during the last trimester of pregnancy. Consequently, it is reduced as soon as they are detected as a preventive practice (Wolfsdorf 2006). This is particularly important in some activities, such as race horses and polo ponies, since it has been demonstrated that twinning rate is heavily influenced by breed (Ginther et al. 1982; Mucha et al. 2012; Riera et al. 2016). Despite twining was pointed as the main cause of chimerism in domestic animals (Padula 2005), the number of cases reported in horses is extremely low (Lear & McGee 2012). Our study, which analyzed three pairs of live foals, showed for the first time that twin foals can be chromosomically normal (non-chimeric) in an certain percentage of cases. Vandeplassche et al. (1970) had already reported in fetuses that most of the cases of chimeric horses were delivered as single births. This fact, along with the absence of symptoms in chimeric horses, could partially explain the lower rate of individuals reported.

Another possible explanation is that paternity tests, which are carried out in most breeders´ associations to determine the correct assignation of the new foals before being accepted in the stud book, are performed analyzing DNA obtained from hair follicles. This sampling procedure is rapidly increasing, instead of the use of blood samples, since samples are easy to obtain and ship to the laboratories, also reducing the stress caused to the animals during collection. However, most of the chimerisms in horses are produced due to a blood exchange between twins during gestation and therefore, they can only be detected using blood-obtained DNA. The fact that the ANCCE still uses blood samples to perform paternity tests in all the individuals could be another explanation for the increased number of animals detected in this study.

Horse karyotyping is a complicated technique, which is performed only in a few laboratories around the world (Lear & Bailey 2008). This and the lack of symptoms of the carrier animals could lead to misdiagnosis of sex chimerism in horses. In this study, we report for the first time a large group of animals showing this kind of genetic abnormality. It is noteworthy that all the animals belong to the same breed and were collected in a time of 8 years. Furthermore, none of the cases reported in this study were sent to the laboratory due to abnormal symptom and they did not show any physical pathology to date. The number of individuals gathered was only possible due the help of breeders and the use of molecular screening tools which allowed to increase and refine the detection rate of this kind of individuals.

Conclusions

Here, we present the most comprehensive study assessing the effect of blood chimerism in horses. All the animals studied were physiologically and morphologically normal and most of them showed a normal reproductive behavior suggesting that sex chimerism in horses is reproductively innocuous. We also describe for the first time a pair of twin foals without cellular chimerism. Our study detected a large number of cases analyzing by molecular techniques individuals of a single breed, suggesting that the number of chimeric horses could be largely underestimated. Further systematic studies are necessary to demonstrate that chimeric horses are reproductively normal.

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Bibliography

Anaya, G., A. Molina, M. Valera, M. Moreno-Millán, P. Azor Ortiz, P. Peral García and S. Demyda-Peyrás (2016). "Chromosomal abnormalities associated with equine infertility: Validation of a simple molecular screening tool in the Purebred Spanish Horse " Animal Genetics.

Anaya, G., M. Moreno-Millán, M. Bugno-Poniewierska, K. Pawlina, A. Membrillo, A. Molina and S. Demyda-Peyrás (2014). "Sex reversal syndrome in the horse: Four new cases of feminization in individuals carrying a 64,XY SRY negative chromosomal complement." Animal Reproduction Science **151**(1-2): 22-27.

Batista, M., F. González, F. Cabrera, E. Palomino, E. Castellano, P. Calero and A. Gracia (2000). "True hermaphroditism in a horned goat with 60XX/60XY chimerism." Canadian Veterinary Journal **41**(7): 562-564.

Brace, M. D., O. Peters, P. Menzies, W. A. King and M. I. Nino-Soto (2008). "Sex chromosome chimerism and the freemartin syndrome in Rideau Arcott sheep." Cytogenet Genome Res **120**(1-2): 132-139.

Bugno-Poniewierska, M., B. Staroń, L. Potocki, A. Gurgul and M. Wnuk (2016). "Identification of unbalanced aberrations in the genome of equine sarcoid cells using CGH technique." Annals of Animal Science **16**(1): 79-85.

Bugno, M., E. Słota and M. Kościelny (2007). "Karyotype evaluation among young horse populations in Poland." Schweizer Archiv fur Tierheilkunde **149**(5): 227-232.

Bugno, M., E. Słota, A. Pieńkowska-Schelling and C. Schelling (2009). "Identification of chromosome abnormalities in the horse using a panel of chromosome-specific painting probes generated by microdissection." Acta Veterinaria Hungarica **57**(3): 369-381.

Bugno, M., E. Słota, M. Tischner and A. Kozubska-Sobocińska (1999). "A case of 64,XX/64,XY leukocytic chimerism in a fertile mare of the Wielkopolska breed." Ann. Anim. Sci. **26**: 9-16.

De Lorenzi, L., V. Genualdo, A. Iannuzzi, G. P. Di Meo, A. Perucatti, R. Mancuso, M. Russo, D. Di Berardino, P. Parma and L. Iannuzzi (2010). "Cytogenetic and genetic studies in a hypospadic horse (Equus caballus, 2n = 64)." Sexual Development **4**(6): 352-357.

Demyda-Peyrás, S., G. Anaya, M. Bugno-Poniewierska, K. Pawlina, A. Membrillo, M. Valera and M. Moreno-Millán (2014). "The use of a novel combination of diagnostic molecular and cytogenetic approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism." Theriogenology **81**(8): 1116-1122.

Demyda-Peyras, S., M. Bugno-Poniewierska, K. Pawlina, G. Anaya and M. Moreno-Millán (2013). "The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: A Case of sex chromosome chimerism in a Spanish Purebred colt." Cytogenetic and Genome Research **141**: 277-283.

Dimsoski, P. (2003). "Development of a 17-plex microsatellite polymerase chain reaction kit for genotyping horses." Croatian Medical Journal **44**(3): 332-335.

Dunn, H. O., D. Smiley, J. R. Duncan and K. McEntee (1981). "Two equine true hermaphrodites with 64,XX/64,XY and 63,XO/64,XY chimerism." The Cornell veterinarian **71**(2): 123-135.

Ginther, O. J., R. H. Douglas and J. R. Lawrence (1982). "Twinning in mares: A survey of veterinarians and analyses of theriogenology records." Theriogenology **18**(3): 333-347.

Hasegawa, T., F. Sato, N. Ishida, Y. Fukushima and H. Mukoyama (2000). "Sex Determination by Simultaneous Amplification of Equine SRY and Amelogenin Genes." Journal of Veterinary Medical Science **62**(10): 1109-1110.

Hinrichs, K., L. C. Buoen and G. R. Ruth (1999). "XX/XY chimerism and freemartinism in a female llama co-twin to a male." Journal of the American Veterinary Medical Association **215**(8): 1140-1141.

Juras, R., T. Raudsepp, P. J. Das, E. Conant and E. G. Cothran (2010). "XX/XY Blood Lymphocyte Chimerism in Heterosexual Dizygotic Twins from an American Bashkir Curly Horse. Case Report." Journal of Equine Veterinary Science **30**(10): 575-580.

Kakoi, H., K. Hirota, H. Gawahara, M. Kurosawa and M. Kuwajima (2005). "Genetic diagnosis of sex chromosome aberrations in horses based on parentage test by microsatellite DNA and analysis of X- and Y-linked markers." Equine Veterinary Journal **37**(2): 143-147.

Lear, T. L. and E. Bailey (2008). "Equine clinical cytogenetics: The past and future." Cytogenetic and Genome Research **120**(1-2): 42-49.

Lear, T. L. and R. B. McGee (2012). "Disorders of sexual development in the domestic horse, Equus caballus." Sexual Development **6**(1-3): 61-71.

McIlwraith, C. W., R. A. Owen and P. K. Basrur (1976). "An equine cryptorchid with testicular and ovarian tissues." Equine veterinary journal **8**(4): 156-160.

Moreno-Millan, M., A. Rodero, F. J. Alonso and A. Sanz (1991). "First report of cytogenetic studies in Spanish breed horses." Genet. Sel. Evol **23**(SUPPL.): 176-178.

Mucha, S., A. Wolc and T. Szwaczkowski (2012). "Bayesian and REML analysis of twinning and fertility in Thoroughbred horses." Livestock Science **144**(1-2): 82-88.

Padula, A. M. (2005). "The freemartin syndrome: An update." Animal Reproduction Science **87**(1-2): 93-109.

Peretti, V., F. Ciotola, S. Albarella, O. Paciello, C. Dario, V. Barbieri and L. Iannuzzi (2008). "XX/XY chimerism in cattle: Clinical and cytogenetic studies." Sexual Development **2**(1): 24-30.

Raudsepp, T., K. Durkin, T. L. Lear, P. J. Das, F. Avila, P. Kachroo and B. P. Chowdhary (2010). "Molecular heterogeneity of XY sex reversal in horses." Anim Genet **41 Suppl 2**(SUPPL. 2): 41-52.

Riera, F. L., J. E. Roldán, J. Gomez and K. Hinrichs (2016). "Factors affecting the efficiency of foal production in a commercial oocyte transfer program." Theriogenology **85**(6): 1053-1062.

Tan, D. K. S. and N. Krekeler (2014). "Success rates of various techniques for reduction of twin pregnancy in mares." Journal of the American Veterinary Medical Association **245**(1): 70-78.

Thomsen, P. D. and P. H. Poulsenm (1993). "Analysis of the Gonadal Sex of Five Intersex Pigs Using Y Chromosomal Markers." Hereditas **119**(3): 205-207.

Vandeplassche, M., L. Podliachouk and R. Beaud (1970). "Some aspects of twin-gestation in the mare." Canadian Journal of Comparative Medicine **34**(3): 218-226.

Wolfsdorf, K. E. (2006). "Management of Postfixation Twins in Mares." Veterinary Clinics of North America - Equine Practice **22**(3): 713-725.

DISCUSIÓN GENERAL

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Muchos de los casos de infertilidad sin una explicación fisiológica aparente en la especie equina están asociados a la presencia de alteraciones cromosómicas. La presente tesis ha confirmado que la mayoría de animales portadores de este tipo de patologías no presentan cambios morfológicos externos o comportamentales hasta alcanzar la madurez sexual, tal y como se ha descrito previamente (Lear and Bailey, 2008).

El 95% de las alteraciones cromosómicas en equinos afectan al par sexual (Lear and McGee, 2012). Las más comunes podrían ser el quimerismo celular, el síndrome de sexo reverso y el síndrome de Turner, abarcando aproximadamente el 90% de los casos publicados (Lear and Bailey, 2008, Lear and McGee, 2012).

El quimerismo celular es una alteración cromosómica caracterizada por la presencia de dos líneas celulares de diferente origen genético en el mismo individuo (Lear and Bailey 2008), bien por la fusión temprana de dos embriones hermanos (quimerismo verdadero) o por el intercambio de células sanguíneas entre los mismos durante la gestación (quimerismo leucocitario). Durante el desarrollo del presente trabajo sólo hemos detectado quimerismo leucocitario o sanguíneo, mostrando que los individuos portadores presentan una apariencia externa normal (Anaya *et al.*, 2017) que les permite aprobar el examen físico que se hace de rutina por el veterinario especializado. Éste síndrome, que se muestra como la patología cromosómica más común en la mayoría de especies ganaderas (Padula, 2005), se considera una alteración rara en caballos, con escasos casos publicados (Demyda-Peyras *et al.*, 2013; 2014). Éste hecho se podría deber principalmente a dos razones: en primer lugar, la gestación gemelar se considera una condición patológica para la yegua y los potros (Miller and Woods 1988), por lo que se eliminaría en la mayoría de los casos uno de los embriones en estadios muy tempranos del desarrollo gestacional antes de que se produzca la anástomosis vascular, impidiendo así, el intercambio celular entre hermanos. En segundo lugar, el quimerismo leucocitario es una enferemedad sin consecuencias reproductivas, por lo

que la inmensa mayoría de los casos no son diagnósticados. Sin embargo, durante el desarrollo del presente estudio se ha logrado detectar el mayor número descrito hasta el momento de caballos quiméricos leucocitarios sin defecto reproductivo alguno.

En contraposición, el síndrome de sexo reverso es uno de los desórdenes ligados al desarrollo sexual (DSD) más comunes en équidos (Villagómez *et al.*, 2011). Los individuos afectados manifiestan un fenotipo sexual opuesto a su sexo cromosómico (yeguas con cariotipos 64,XY y machos con cariotipos 64,XX). No obstante, existen tres formas de presentación diferentes de éste síndrome en base al cariotipo y presencia o no del gen *SRY*. Éste, descrito por Sinclair *et al.* (1990), permite, al activarse, la sobreexpresión de una cascada de genes autosómicos (*SOX9* y *SF1* entre otros) que desencadenan la liberación de la hormona anti Mülleriana (AMH), evitando el desarrollo ovárico y por consiguiente, el fenotipo femenino (De Santa Barbara *et al.*, 1998). De esta manera se han descrito en la especie equina los machos 64, XX; *SRY* negativo y las hembras 64, XY *SRY* positivo o negativo (Villagómez *et al.*, 2011). Aún no se ha descrito ningún caso de macho 64,XX *SRY* positivo en caballo a diferencia de en humanos (Abbas *et al.*, 1993). Dentro de este síndrome, las hembras 64, XY *SRY* negativo representan el caso de mayor incidencia hasta la fecha (Favetta *et al.*, 2012), proponiendo que está causado por una deleción parcial de un segmento del cromosoma ECAY en el que se localiza el gen *SRY* (Raudsepp *et al.*, 2010) evitando desarrollar, por consiguiente, su acción biológica. Estos animales, a diferencia de los otros dos casos que manifiestan graves anomalías morfológicas (Ciotola *et al.*, 2012, Révay *et al.*, 2012), suelen presentar genitales externos normales, aunque en la mayoría de los casos muestran cierto grado de disgenesia gonadal, principalmente ovárica, asociada con una fertilidad reducida (Lear and McGee, 2012).

El síndrome de Turner (63,X) es la alteración cromosómica más documentada en caballos (Lear and McGee, 2012). Los animales que la manifiestan tienen una conformación de yegua normal sin anomalías visibles en los órganos reproductores externos aunque en algunos casos, los individuos pueden presentar una apariencia masculinizada (Bugno *et al.*, 2008).

La presencia de éste tipo de alteraciones suponen un hándicap para el ganadero dedicado a la cría. Animales con una apariencia externa aparentemente normal comienzan a manifestar problemas reproductivos al alcanzar la madurez sexual. Tras haber utilizado a los reproductores en varios ciclos sin éxito y descartadas otras posibles causas (pe. infecciosas) es posible orientar su diagnóstico hacia este tipo de patologías genéticas. Debido a esto, los animales que presentan estas patologías no podrán ser utilizados como reproductores, lo que disminuye considerablemente su valor. El gasto de tiempo y dinero que supone el mantenimiento de estos individuos no aptos para la cría puede ser un hecho relevante para la supervivencia y viabilidad de muchas ganaderías, más aun teniendo en cuenta que la infertilidad provocada por alteraciones cromosómicas no es reversible ni tiene tratamiento alguno.

A la dificultad para detectar este tipo de animales por la ausencia de alteraciones morfológicas, hay que añadir un problema de carácter técnico: la detección de este tipo de alteraciones se realiza tradicionalmente mediante el cariotipado de los individuos a partir de metafases obtenidas de cultivos leucocitario (Moreno-Millan *et al.*, 1991), técnica muy difícil y costosa debido a la complejidad del cariotipo del caballo, lo que hace que pocos laboratorios a nivel mundial sean capaces de desarrollarlas (Lear and Bailey, 2008). Por esta misma razón la cantidad de animales que quedan sin diagnosticar en edades tempranas puede ser elevada.

Actualmente la aparición de técnicas de biología molecular ha revolucionado tanto el desarrollo científico como las metodologías diagnósticas. Estas metodologías, caracterizadas por su rapidez, sencillez y elevada posibilidad de automatización, han presentado un enorme desarrollo como complemento de las técnicas diagnósticas clásicas tanto en el ser humano (Munné, 2012) como en distintas especies animales (Gurgul *et al.*, 2014, Raudsepp and Chowdhary, 2016). Entré ellas, los marcadores de tipo microsatélite (*short tándem repeat makers*; STR´s) se han utilizado en innumerables aplicaciones científicas y forenses debido a su elevada variabilidad, fiabilidad y posibilidad de automatización. En particular, la ANNCE utiliza un panel de 18 marcadores microsatélites (17 STRs localizados en cromosomas autosómicos y 1 STR localizado en el ECAX) para pruebas de exclusión de paternidad. El genotipado de los reproductores con este panel de marcadores puede suponer una herramienta útil para la detección de animales

quiméricos (Demyda-Peyras *et al.*, 2013). La detección es posible cuando, al analizar la muestra que contiene las dos poblaciones celulares de origen genético distinto, aparecen 3 o 4 alelos en al menos un STR, algo imposible de darse en organismos diploides sin ningún tipo de alteración cromosómica.

La fiabilidad de un panel de marcadores utilizado para exclusión de paternidad suele medirse mediante el parámetro probabilidad de identidad (PID), que se refiere a la probabilidad de que dos individuos escogidos al azar compartan el mismo perfil genético (Taberlet and Luikart, 1999), que depende de las frecuencias alélicas de los diferentes microsatélites utilizados en dicha población así como el número de marcadores empleados. Cuanto menor sea dicha probabilidad mayor será la fiabilidad del test No obstante, la probabilidad de detectar un caso de quimerismo viene determinada por la probabilidad de que dos hermanos completos presenten exactamente el mismo perfil genético cuando se usa dicho panel de microsatélites (probabilidad de identidad entre hermanos; PID_{sib}; Waits *et al.*, 2001; Eichmann *et al.*, 2005). El panel de marcadores utilizado por la ANCCE tiene una PID_{sib} igual a 0,00000088, lo que implica que en casi la totalidad de los casos (99,99%) podremos detectar un caso de quimerismo a partir de una muestra que contenga las líneas celulares de los dos hermanos debido a la aparición de 3 o 4 alelos en al menos uno de los 17 marcadores autosómicos. Sin embargo, en quimeras de tipo hematopoyético, también se puede hacer uso del análisis comparativo de muestras de ADN obtenidas a partir de tejido sanguíneo y de muestras de ADN obtenidas de otro tejido (normalmente de folículos pilosos) del mismo individuo. En este caso, los resultados obtenidos a partir del ADN "sanguíneo" presentará las dos líneas celulares (aparecerá al menos un marcador con 3 o 4 alelos) mientras que la muestra de ADN de "folículos pilosos" sólo presentará la línea celular del feto original (nunca aparecerá un marcador con más de 2 alelos) mostrando una diferencia entre ambos perfiles genéticos.

En base a éste principio, esta tesis ha demostrado que las actuales pruebas de filiación pueden ser una herramienta complementaria de gran utilidad en el diagnóstico de quimeras (Demyda-Peyras *et al.*, 2013, Demyda-Peyrás *et al.*, 2014). Sin embargo este test no es una herramienta válida para la detección del resto de alteraciones que afectan a los cromosomas sexuales debido a que el panel habitualmente utilizado presenta únicamente un marcador del ECAX (*LEX003*). En éste sentido un síndrome de Turner se

diagnosticaría en el 100% de los casos con una fiabilidad de tan sólo el 19,8 % lo que supondría que todos los animales con la patología se detectarían mientras que una de cada 5 yeguas normales se describirían como afectadas (falso positivo). Esta probabilidad sería idéntica en la detección de Yeguas con sexo reverso, 64XY. Por tanto, además de ofrecer una fiabilidad baja, la técnica no podría discriminar entre estas dos alteraciones. Por otro lado, los machos sexo reverso, 64XX, se diagnosticarían en el 100% de los casos con una fiabilidad del 80,2%, dejando pasar un 19,8% de animales positivos con lo que uno de cada 5 animales con esta afección no sería detectado.

Por lo tanto para la detección mediante técnicas moleculares de las alteraciones que implican a los cromosomas sexuales es necesario ampliar el número de marcadores moleculares específicos del ECAX y el ECAY. La inclusión de los fragmentos del gen codificante de las proteínas de "dedos de zinc" (*ZFX* y *ZFY*) o del gen codificante de la amelogenina (*AMX* y *AMY*), permite conocer la presencia o ausencia de los cromosomas sexuales, así como determinar el sexo genético de los individuos. Su uso se convierte en crítico para el diagnóstico de los sexos reversos al mostrar una dotación de los cromosomas sexuales opuesta al fenotipo del animal permitiendo la detección de yeguas XY y caballos XX con una fiabilidad del 100% (Demyda-Peyras *et al.*, 2013). A su vez, el análisis de la región específica del gen de determinación del sexo Y (*SRY*) permite la discriminación entre yeguas, XY, SRY positivo o negativo en función de la amplificación o no de dicho fragmento. Sin embargo, si bien todos los casos de síndrome de Turner se detectarían con esta ampliación de marcadores, no habría manera de discriminar entre una hembra cromosómica normal y un animal Turner. La utilización del marcador del panel de exclusión de paternidad *LEX003*, permite la detección del 100% de las yeguas con síndrome de Turner, bajando el número de falsos positivos a un 19,8% (probabilidad de que se presente el marcador *LEX003* en homocigosis, teniendo AMEX positiva y AMEY negativa). Por tanto, aunque el uso de los marcadores moleculares de los cromosomas sexuales de uso habitual presentan cierta utilidad para el diagnóstico de alteraciones de tipo cromosómico, su fiabilidad para el diagnóstico de rutina no es suficiente. Dado que el objetivo central de la presente Tesis Doctoral ha sido diseñar, implementar y validar un sistema diagnóstico basado en técnicas de biología molecular, rápido y eficaz para la detección precoz de las

principales alteraciones cromosómicas que generan infertilidad en la especie equina, que sirva como técnica sustitutiva y/o complementaria a las técnicas clásicas de diagnóstico (técnicas citogenéticas de cariotipado y bandeo) ha sido necesario aumentar el número de marcadores para ampliar la fiabilidad en dicha detección.

Para ello se puso a punto y se seleccionó un grupo de marcadores moleculares representativos de los cromosomas sexuales en caballo, estimándose la capacidad de detección para las alteraciones anteriormente descritas en caballos. Todo ello se validó a nivel estadístico utilizando como patrón resultados previamente obtenidos mediante las técnicas citogenéticas.

Este panel de marcadores ampliado fue implementado en una sola reacción de PCR, permitiendo por lo tanto el diagnóstico de las patologías cromosómicas más importantes en esta especie (Quimerísmo, síndrome de sexo reverso y síndrome de Turner) mediante un test rápido y muy económico. Este panel incluía 5 STR´s localizados en el cromosoma ECAX (*LEX003*, *LEX026*, *TKY38*, *TKY270* y *UCDEQ502*) y 1 STR y un fragmento monomórfico localizados en el cromosoma ECAY (*YH12* y *SRY* respectivamente). En este estudio, los resultados obtenidos mediante el análisis molecular coincidieron en un 100% con el diagnóstico derivado previamente de las técnicas citogenéticas, lo que permitió la validación del método como herramienta diagnostica.

La fiabilidad de toda herramienta diagnostica se caracteriza por su sensibilidad y especificidad (Van Der Schouw *et al.*, 1995). En el caso de los test de cribado, que se utilizan para estimar de manera rápida y simple la prevalencia de alguna enfermedad en una población, es importante que posean una elevada sensibilidad para evitar dar falsos negativos, lo que provocaría que animales portadores quedasen sin diagnóstico. En nuestro caso es igualmente importante evitar los falsos positivos que podría suponer descartar para la reproducción individuos completamente sanos. En las herramientas diagnósticas basadas en el uso de marcadores moleculares la fiabilidad viene derivada del valor del parámetro índice de variabilidad o PIC (*polymorphism information content*) que es proporcional al número de marcadores

empleados y al polimorfismo de los mismos (número de alelos y frecuencia de los mismos) (Garvin *et al.*, 1998).

El panel de marcadores moleculares desarrollado se caracterizó en una población de caballos de Pura Raza Española. Para ello, se genotiparon 271 animales pertenecientes a 90 ganaderías diferentes, seleccionando los animales menos emparentados presentes en el libro genealógico de PRE. Los marcadores empleados en éste estudio han demostrado ser altamente polimórficos con PICs comprendidos entre 0,63 y 0,8. Como consecuencia de esto hemos determinado la existencia de un número de posibles genotipos diferentes en torno a 745 millones y un PIC global de 0,68. Los valores de PIC de los marcadores empleados calculados sobre el caballo de Pura Raza Español, no difiere del observado en marcadores de distintas razas japonesas, (Tozaki *et al.*, 2003), europeas (Achmann *et al.*, 2004) y españolas (Cañon *et al.*, 2000) lo que sugiere que esta metodología mantendría su fiabilidad dignóstica (valores de sensibilidad y especificidad) independientemente de la raza analizada.

Metodológicamente, la herramienta desarrollada permite generar 10 tipos de perfiles moleculares distintos en base a la combinación de los resultados obtenidos en los diferentes tipos de marcadores (Tabla1). Así su análisis permite diferenciar entre machos y hembras cromosómicamente normales; síndromes de Turner (64,X0); quimerismo celular hembra-macho (64,XX/64,XY); quimerismo celular macho-macho (64,XY/64,XY); quimerismo celular hembra-hembra (64,XX/64,XX); síndromes de sexo reverso en hembras (64 XY,) *SRY* positivo o negativo y síndromes de sexo reverso en macho (64,XX) *SRY* positivo o negativo.

Si el genotipado de un animal determinase la aparición de un solo alelo en todos los marcadores del ECAX y amplificación en los marcadores del ECAY, el diagnóstico sería el de un macho cromosómicamente normal con un 100% de sensibilidad y especificidad. De la misma forma si apareciese al menos un marcador del ECAX en heterocigosis y ausencia de amplificación en los marcadores del ECAY estaríamos frente a un perfil de hembra cromosómicamente normal. En este caso se alcanzaría una sensibilidad del 99,82% ya que el 0,18% (probabilidad de presentar todos los marcadores del cromosoma

X en homocigosis) de las hembras normales (1 de cada 588) serían diagnosticadas erróneamente como un animal con síndrome de Turner (falso positivo). La especificidad se situaría en un 100%.

El perfil de los animales con síndrome de Turner mostraría solo un alelo por marcador en todos los STRs del ECAX y ausencia de amplificación en los marcadores del ECAY. En éste caso todo animal afectado con síndrome de Turner se identificaría correctamente como positivo (sensibilidad del 100%) y mientras que habría posibilidad de dar un resultado de falso negativo generando un valor de especificidad del 99,82%.

Asimismo, el panel puesto a punto permite un diagnóstico fácil del síndrome de sexo reverso ya que el perfil genético obtenido será compatible con el sexo opuesto al sexo fenotípico. Así las yeguas XY sexo reverso presentarán un solo alelo en cada marcador del ECAX, amplificación en el marcador EcaYH12, y amplificación o no en el marcador *SRY*. Todos los animales afectados se diagnosticarán ofreciendo una sensibilidad y una especificidad del 100%.

Los machos XX sexo reverso *SRY* negativos presentarán un perfil genético de yegua normal, es decir, al menos uno de los 5 marcadores del ECAX aparecerá en heterocigosis (con una probabilidad del 99,82%) y no habrá amplificación en los marcadores del ECAY. Así todos los animales afectados se diagnosticarán como animales con alteración (sensibilidad 100%). Sin embargo, un 0,18% de los casos positivos se diagnosticarán como síndrome de Turner (especificidad 99,82%). Aún no se han descrito los machos XX sexo reverso *SRY* positivo, pero sería detectado de forma teórica por nuestra herramienta. El perfil generado presentaría al menos un marcador del ECAX en heterocigosis junto con amplificación en el marcador *SRY* con una sensibilidad y especificidad del 100%.

Finalmente, la capacidad de detección de quimeras del nuevo panel viene determinado por su valor de $PID_{(sib)}$ igual a 0,0129 (existe un 1,3% de probabilidad de que dos hermanos completos tengan el mismo perfil genético). No obstante, los animales quiméricos pueden originarse entre hermanos del mismo o distinto sexo lo que determinaría un valor diferente de este parámetro por la diferencia entre polimorfismos en los marcadores del ECAX y ECAY. Las quimeras que poseen dos líneas celulares cuyo

origen sean hermanos de distinto sexo presentarán amplificación en los marcadores del ECAY, además de presentar al menos en un marcador del ECAX, 2 o 3 alelos. La fiabilidad del diagnóstico determinada por el valor de $PI_{(sib)}$ junto con la probabilidad de que la línea celular de hembra no sea homocigota en todos sus marcadores del ECAX, permite una especificidad del 99,99%. En el caso quimeras cuyo origen son dos hermanas ésta especificidad se sitúa en un 98,35%. Si analizásemos una muestra de quimera con dos líneas celulares de macho, la capacidad de detección se situaría en un 98,71%. Adicionalmente ésta metodología permite diferenciar entre quimeras en base a su origen Así, si al analizar muestras de otros tejidos existen diferencias entre los perfiles nos encontraríamos ante una quimera hematopoyetica, siendo en caso contrario un caso de quimera verdadera. Esta distinción cobra especial importancia puesto que mientras las quimeras verdaderas presentan órganos reproductivos aberrantes, tanto externos como internos (Batista *et al.*, 2000, Brace *et al.*, 2008), el efecto del quimerismo de origen sanguíneo sigue aún bajo debate. Así mientras autores como Juras et al. (2010) han sugerido que es inocuo en los aspectos reproductivos del animal, otros como Bugno et al. (1999) han descrito el caso de una yegua quimérica con una reducción de la fertilidad sin determinar la causa, o como Moreno-Millan et al. (1991) publicaron un caso en el que la hembra era estéril mostrando ovarios hipoplásicos y falta de ciclo. Nuestros resultados muestran que 15 casos de quimerismo hematopoyético de los 16 analizados en esta tesis (Demyda *et al*, 2011, Anaya *et al.*, 2017), pueden tener descendencia (si bien quedaría por determinar si con una fertilidad reducida o no).

Tabla1: Perfiles moleculares del panel desarrollado con sus correspondientes valores de Sensibilidad y Especificidad.

Fenotipo	Genotipo Molecular			Diagnóstico Molecular	Sensibilidad	Especificidad
	Perfil de STRs del ECAX	Marcadores del ECAY				
		ECAYH12	*SRY*			
Macho	Un alelo por marcador	+	+	Macho normal	100%	100%
Hembra	Al menos un marcador en heterocigosis	-	-	Hembra normal	99,82%	100%
Hembra	Un alelo por marcador	-	-	Síndrome de Turner	100%	99,82%
Macho	Al menos una marcador con 2 alelos	+	+	Macho quimérico	100%	98,71%
Hembra	Al menos un marcador con 3 o 4 alelos	-	-	Hembra quimérica	100%	98,35%
Macho	Al menos un marcador con 2 o 3 alelos	+	+	Macho quimérico	100%	99,99%
Hembra	Al menos un marcador con 2 o 3 alelos	+	+	Hembra quimérica	100%	99,99%
Macho	Al menos un marcador en heterocigosis	-	+	Sexo reverso, Macho SRY positivo	100%	100%
Macho	Al menos un marcador en heterocigosis	-	-	Sexo reverso, Macho SRY negativo	100%	100%
Hembra	Un alelo por marcador	+	+	Sexo reverso, Hembra SRY positivo*	100%	100%
Hembra	Un alelo por marcador	+	-	Sexo reverso, Hembra SRY negativo	99,82%	100%

*No se ha descrito en caballos ningún caso de Sexo reverso, Hembra SRY positivo.

Una vez puesta a punto la herramienta molecular y validada nos propusimos su utilización para intentar obtener una estima de la prevalencia en la población de PRE de las anteriormente descritas alteraciones cromosómicas. Estudios de otros autores en la especie equina en los que se incluía esta raza, mostraron una prevalencia global del 2% (Bugno *et al.*, 2009). Sin embargo, en nuestro caso, de los 271 animales analizados, el 100% presentaron un genotipo normal. Esta gran diferencia podría deberse al hecho de que animales con problemas de infertilidad suelen darse de baja del libro genealógico sin que se determine la causa, al no ser habitual el diagnóstico genético en ésta raza. Otra posible causa podría deberse a que la prevalencia de las aberraciones cromosómicas en la raza estudiada sea sensiblemente menor a la descrita por Bugno *et al.* (2007). En éste sentido, con una prevalencia menor a un 1%, podría no aparecer ningún caso positivo por puro azar en la población analizada.

Según los resultados obtenidos en la presente tesis y haciendo una evaluación global de los mismos se ha podido comprobar que los marcadores moleculares son de gran utilidad en la detección de alteraciones cromosómicas en équidos. La integración de un conjunto de marcadores específicos de los cromosomas sexuales junto con genes relacionados con la determinación sexual permite el diagnóstico del 95% de éste tipo de patologías en esta especie con una sensibilidad entre 99,82% y 100% y una especificidad entre 98,35% y 100%. Los resultados del diagnóstico molecular han coincidido con los resultados del diagnóstico citogenético, validando por tanto la técnica. De ésta manera, la implantación como herramienta de rutina en potros al nacimiento podría suponer una herramienta muy útil para descartar aquellos animales que no van a ser aptos para la reproducción de una manera muy eficiente, económica y precoz, con el consiguiente ahorro en tiempo y dinero para los productores.

CONCLUSIONES

CONCLUSIONES

Durante el desarrollo de la presente tésis doctoral se ha podido llegar a las siguientes conclusiones:

1- El presente trabajo ha logrado desarrollar una herramienta molecular que permite la detección fiable de las principales anomalías cromosómicas de la especie equina. Dicha metodología ha generado la patente española titulada "MÉTODO PARA EL DAGNÓSTICO PRECOZ DE LA INFERTILIDAD EQUINA" con número P201630755.

2- La integración de un grupo específico de marcadores moleculares de tipo STR junto con un fragmento relacionado con la determinación sexual en una sola reacción de PCR, permite un diagnóstico muy rápido y económico en relación a las técnicas citogenéticas tradicionales.

3- Según nuestros resultados, la prevalencia de este tipo de patologías en los reproductores de Pura Raza Español es muy baja (inferior al 1%).

4- Esta tesis contiene el mayor número de casos descritos de quimerismo leucocitario hasta la actualidad a nivel mundial, incluyendo por primera vez la descripción de 4 pares de hermanos quiméricos hematopoyéticos vivos.

5- De la misma forma, el presente trabajo contiene la descripción del primer caso a nivel mundial en la especie equina de quimerismo leucocitario en el que una de las líneas celulares es cromosómicamente aberrante (64,XY/63,X0).

6- En éste trabajo se ha demostrado que, a diferencia del quimerismo completo, el leucocitario no está ligado a la infertilidad en los equinos, por lo que la diferenciación de ambos tipos cobra una elevada importancia en ésta especie.

7- Aunque esta herramienta ha sido desarrollada y validada específicamente para la población de PRE, el análisis preliminar de las frecuencias alélicas de los marcadores en otras razas indica que ésta herramienta puede ser utilizada en otras poblaciones equinas sin perder fiabilidad.

8- El uso de la metodología planteada en la presente tesis como una herramienta de diagnóstico de rutina en los programas de cría podría permitir la detección de forma sistemática, temprana y eficiente de la mayoría de las alteraciones cromosómicas asociadas a la infertilidad en la especie equina permitiendo el ahorro de tiempo y dinero a los criadores.

REFERENCIAS BIBLIOGRÁFICAS

REFERENCIAS BIBLIOGRÁFICAS GENERALES

Abbas, N., K. McElreavey, M. Leconiat, E. Vilain, F. Jaubert, R. Berger, C. Nihoul-Fekete, R. Rappaport and M. Fellous (1993). "Familial case of 46,XX male and 46,XX true hermaphrodite associated with a paternal-derived SRY-bearing X chromosome." Comptes Rendus de l'Academie des Sciences - Series III **316**(4): 375-383.

Abe, S., Y. I. Miyake, S. I. Kageyama, G. Watanabe, K. Taya and K. Kawakura (1999). "Deletion of the Sry region on the Y chromosome detected in a case of equine gonadal hypoplasia (XY female) with abnormal hormonal profiles." Equine Vet J **31**(4): 336-338.

Achmann, R., I. Curik, P. Dovc, T. Kavar, I. Bodo, F. Habe, E. Marti, J. Sölkner and G. Brem (2004). "Microsatellite diversity, population subdivision and gene flow in the Lipizzan horse." Animal Genetics **35**(4): 285-292.

Affara, N. A., I. J. Chalmers and M. A. Ferguson-Smith (1993). "Analysis of the SRY gene in 22 sex-reversed XY females identifies four new point mutations in the conserved DNA binding domain." Human Molecular Genetics **2**(6): 785-789.

Anaya, G., M. Moreno-Millán, M. Bugno-Poniewierska, K. Pawlina, A. Membrillo, A. Molina and S. Demyda-Peyrás (2014). "Sex reversal syndrome in the horse: Four new cases of feminization in individuals carrying a 64,XY SRY negative chromosomal complement." Animal Reproduction Science **151**(1-2): 22-27.

Anaya, G., A. Molina, M. Valera, M. Moreno-Millán, P. Azor Ortiz, P. Peral García and S. Demyda-Peyrás (2016). "Chromosomal abnormalities associated with equine infertility: Validation of a simple molecular screening tool in the Purebred Spanish Horse " Animal Genetics.

Anaya, G., M. Valera, A. Molina, P. Azor, M. Moreno-Millán, M. E. Fernandez, M. Solé, S. Negro and S. Demyda-Peyrás (2017). "Blood chimerism in the horse is reproductively innocuous? a 15 cases report." *In preparation*.

Anne, S. J. (2009). "Diagnosis and management off twinning in mares." In Practice **31**(5): 226-231.

Aurich, J. E. (2012). "Artificial Insemination in Horses-More than a Century of Practice and Research." Journal of Equine Veterinary Science **32**(8): 458-463.

Bannasch, D., C. Rinaldo, L. Millon, K. Latson, T. Spangler, S. Hubberty, L. Galuppo and L. Lowenstine (2007). "SRY negative 64,XX intersex phenotype in an American saddlebred horse." Veterinary Journal **173**(2): 437-439.

Batista, M., F. González, F. Cabrera, E. Palomino, E. Castellano, P. Calero and A. Gracia (2000). "True hermaphroditism in a horned goat with 60XX/60XY chimerism." Canadian Veterinary Journal **41**(7): 562-564.

Belkhir, K., P. Borsa, L. Chikhi, N. Raufaste and F. Bonhomme (1996-2004). GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations, Laboratoire Génome, Populations, Interactions, CNRS UMR 5171.

Berta, P., J. R. Hawkins, A. H. Sinclair, A. Taylor, B. L. Griffiths, P. N. Goodfellow and M. Fellous (1990). "Genetic evidence equating SRY and the testis-determining factor." Nature **348**(6300): 448-450.

Bjørnstad, G. and K. H. Røed (2002). "Evaluation of factors affecting individual assignment precision using microsatellite data from horse breeds and simulated breed crosses." Animal Genetics **33**(4): 264-270.

Brace, M. D., O. Peters, P. Menzies, W. A. King and M. I. Nino-Soto (2008). "Sex chromosome chimerism and the freemartin syndrome in Rideau Arcott sheep." Cytogenet Genome Res **120**(1-2): 132-139.

Bouters, R. and M. Vandeplassche (1972). "Twin gestation in the mare: the incidence of placental vascular anastomoses and their influence on the reproductive performance of heterosexual equine twins." Journal of Reproduction and Fertility **29**(1): 149.

Bowling, A. T., M. Breen, B. P. Chowdhary, K. Hirota, T. Lear, L. V. Millon, F. A. De Ponce Leon, T. Raudsepp and G. Stranzinger (1997). "Report of the Third International Committee for the standardization of the domestic horse Karyotype, Davis, CA, USA, 1996. International system for cytogenetic nomenclature of the domestic horse." Chromosome Research **5**(7): 433-443.

Bowling, A. T., L. Millon and J. P. Hughes (1987). "An update of chromosomal abnormalities in mares." J Reprod Fertil Suppl **35**: 149-155.

Bowling, A. T., M. L. Stott and L. Bickel (1993). "Silent blood chimaerism in a mare confirmed by DNA marker analysis of hair bulbs." Animal Genetics **24**(4): 323-324.

Breen, M., C. F. Langford, N. P. Carter, P. E. Fischer, E. Marti, C. Gerstenberg, W. R. Allen, T. L. Lear and M. M. Binns (1997). "Detection of equine X chromosome abnormalities in equids using a horse X whole chromosome paint probe (WCPP)." Veterinary Journal **153**(3): 235-238.

Bugno-Poniewierska, M., B. Staroń, L. Potocki, A. Gurgul and M. Wnuk (2016). "Identification of unbalanced aberrations in the genome of equine sarcoid cells using CGH technique." Annals of Animal Science **16**(1): 79-85.

Bugno, M., J. Klukowska, E. Słota, M. Tischner and M. Świtoński (2003). "A sporadic case of the sex-reversed mare (64,XY; SRY-negative): Molecular and cytogenetic studies of the Y chromosome." Theriogenology **59**(7): 1597-1603.

Bugno, M. and E. Słota (2007). "Application of arm-specific painting probes of horse X chromosome for karyotype analysis in an infertile Hutsul mare with 64,XX/65,XX + XP karyotype: Case report." Acta Veterinaria Hungarica **55**(3): 309-314.

Bugno, M., E. Słota and M. Kościelny (2007). "Karyotype evaluation among young horse populations in Poland." Schweizer Archiv fur Tierheilkunde **149**(5): 227-232.

Bugno, M., E. Słota, A. Pieńkowska-Schelling and C. Schelling (2007). "Detection of equine X cromosome mosaicism in a mare using an equine X whole chromosome painting probe (WCPP) - A case report." Acta Veterinaria Hungarica **55**(2): 207-212.

Bugno, M., E. Słota, A. Pieńkowska-Schelling and C. Schelling (2009). "Identification of chromosome abnormalities in the horse using a panel of chromosome-specific painting probes generated by microdissection." Acta Veterinaria Hungarica 57(3): 369-381.

Bugno, M., T. Ząbek, P. Golonka, A. Pieńkowska-Schelling, C. Schelling and E. Słota (2008). "A case of an intersex horse with 63,X/64,XX/65,XX,del(Y)(q?) karyotype." Cytogenetic and Genome Research 120(1-2): 123-126.

Cañon, J., M. L. Checa, C. Carleos, J. L. Vega-Pla, M. Vallejo and S. Dunner (2000). "The genetic structure of Spanish Celtic horse breeds inferred from microsatellite data." Animal Genetics **31**(1): 39-48.

Chandley, A. C., J. Fletcher, P. D. Rossdale, C. K. Peace, S. W. Ricketts, R. J. McEnery, J. P. Thorne, R. V. Short and W. R. Allen (1975). "Chromosome abnormalities as a cause of infertility in mares." Journal of reproduction and fertility. Supplement(23): 377-383.

Cheung, S. W., C. A. Shaw, D. A. Scott, A. Patel, T. Sahoo, C. A. Bacino, A. Pursley, J. Li, R. Erickson, A. L. Gropman, D. T. Miller, M. R. Seashore, A. M. Summers, P. Stankiewicz, A. C. Chinault, J. R. Lupski, A. L. Beaudet and V. R. Sutton (2007). "Microarray-based CGH detects chromosomal mosaicism not revealed by conventional cytogenetics." American Journal of Medical Genetics, Part A **143**(15): 1679-1686.

Ciotola, F., S. Albarella, M. P. Pasolini, L. Auletta, L. Esposito, L. Iannuzzi and V. Peretti (2012). "Molecular and cytogenetic studies in a case of XX SRY -Negative sex reversal in an Arabian horse." Sexual Development **6**(1-3): 104-107.

Coogle, L., E. Bailey, R. Reid and M. Russ (1996). "Equine dinucleotide repeat polymorphisms at loci LEX002, -003, -004, -005, -007, -008, -009, -010, -011, -013 and -014." Animal genetics **27**(2): 126-127.

Coogle, L., R. Reid and E. Bailey (1996). "Equine dinucleotide repeat loci from LEX025 to LEX033." Animal Genetics **27**(4): 289-290.

Davies Morel, M. C. G., J. R. Newcombe and M. Lauber (2012). "Manual reduction of multiple embryos in the mare: The effect on subsequent pregnancy outcome." Veterinary Journal **192**(3): 322-325.

Di Meo, G. P., G. Neglia, A. Perucatti, V. Genualdo, A. Iannuzzi, D. Crocco, D. Incarnato, G. Romano, P. Parma and L. Iannuzzi (2009). "Numerical sex chromosome aberrations and abnormal sex development in horse and sheep." Sexual development : genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation **3**(6): 329-332.

Dimsoski, P. (2003). "Development of a 17-plex microsatellite polymerase chain reaction kit for genotyping horses." Croatian Medical Journal **44**(3): 332-335.

De Lorenzi, L., E. Rossi, V. Genualdo, S. Gimelli, E. Lasagna, A. Perucatti, A. Iannuzzi and P. Parma (2012). "Molecular characterization of Xp chromosome deletion in a fertile cow." Sexual Development **6**(6): 298-302.

De Santa Barbara, P., N. Bonneaud, B. Boizet, M. Desclozeaux, B. Moniot, P. Sudbeck, G. Scherer, F. Poulat and P. Berta (1998). "Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene." Molecular and Cellular Biology **18**(11): 6653-6665.

De Lorenzi, L., V. Genualdo, A. Iannuzzi, G. P. Di Meo, A. Perucatti, R. Mancuso, M. Russo, D. Di Berardino, P. Parma and L. Iannuzzi (2010). "Cytogenetic and genetic studies in a hypospadic horse (Equus caballus, 2n = 64)." Sexual Development **4**(6): 352-357.

Demyda-Peyrás, S., G. Anaya, M. Bugno-Poniewierska, K. Pawlina, A. Membrillo, M. Valera and M. Moreno-Millán (2014). "The use of a novel combination of diagnostic molecular and cytogenetic approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism." Theriogenology **81**(8): 1116-1122.

Demyda-Peyras, S., M. Bugno-Poniewierska, K. Pawlina, G. Anaya and M. Moreno-Millán (2013). "The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: A Case of sex chromosome chimerism in a Spanish Purebred colt." Cytogenetic and Genome Research **141**: 277-283.

Dimsoski, P. (2003). "Development of a 17-plex microsatellite polymerase chain reaction kit for genotyping horses." Croatian Medical Journal **44**(3): 332-335.

Donaghue, C., K. Mann, Z. Docherty, R. Mazzaschi, C. Fear and C. Ogilvie (2010). "Combined QF-PCR and MLPA molecular analysis of miscarriage products: An efficient and robust alternative to karyotype analysis." Prenatal Diagnosis **30**(2): 133-137.

Dunn, H. O., D. Smiley, J. R. Duncan and K. McEntee (1981). "Two equine true hermaphrodites with 64,XX/64,XY and 63,XO/64,XY chimerism." Cornell Vet **71**(2): 123-135.

Edwards, S. and J. J. Waters (2008). "Prenatal diagnosis of monosomy 18p involving a jumping translocation." Prenatal Diagnosis **28**(8): 764-766.

Eggleston-Stott, M. L., A. DelValle, M. Bautista, S. Dileanis, E. Wictum and A. T. Bowling (1997). "Nine equine dinucleotide repeats at microsatellite loci UCDEQ136, UCDEQ405, UCDEQ412, UCDEQ425, UCDEQ437, UCDEQ467, UCDEQ487, UCDEQ502 and UCDEQ505." Animal Genetics **28**(5): 370-371.

Eichmann, C., B. Berger, M. Steinlechner and W. Parson (2005). "Estimating the probability of identity in a random dog population using 15 highly polymorphic canine STR markers." Forensic Science International **151**(1): 37-44.

Ergun-Longmire, B., G. Vinci, L. Alonso, S. Matthew, S. Tansil, K. Lin-Su, K. McElreavey and M. I. New (2005). "Clinical, hormonal and cytogenetic evaluation of 46,XX males and review of the literature." Journal of Pediatric Endocrinology and Metabolism **18**(8): 739-748.

Favetta, L. A., D. A. F. Villagómez, L. Iannuzzi, G. Di Meo, A. Webb, S. Crain and W. A. King (2012). "Disorders of sexual development and abnormal early development in domestic food-producing mammals: The role of chromosome abnormalities, environment and stress factors." Sexual Development **6**(1-3): 18-32.

Ferguson-Smith, M. A. (1965). "Karyotype-phenotype correlations in gonadal dysgenesis and their bearing on the pathogenesis of malformations." Journal of medical genetics **39**: 142-155.

Fernández, M. E., D. E. Goszczynski, J. P. Lirón, E. E. Villegas-Castagnasso, M. H. Carino, M. V. Ripoli, A. Rogberg-Muñoz, D. M. Posik, P. Peral-García and G. Giovambattista (2013). "Comparison of the effectiveness of microsatellites and SNP panels for genetic identification, traceability and assessment of parentage in an inbred Angus herd." Genetics and Molecular Biology **36**(2): 185-191.

Ford, C. E., K. W. Jones, P. E. Polani, J. C. De Almeida and J. H. Briggs (1959). "A sex-chromosome anomaly in a case of gonadal dysgenesis (Turner's syndrome)." The Lancet **273**(7075): 711-713.

Gall, J. G. and M. L. Pardue (1969). "Formation and detection of RNA-DNA hybrid molecules in cytological preparations." Proceedings of the National Academy of Sciences of the United States of America **63**(2): 378-383.

Garvin, A. M., W. Holzgreve and S. Hahn (1998). "Highly accurate analysis of heterozygous loci by single cell PCR." Nucleic Acids Research **26**(15): 3468-3472.

Ginther, O. J., R. H. Douglas and J. R. Lawrence (1982). "Twinning in mares: A survey of veterinarians and analyses of theriogenology records." Theriogenology **18**(3): 333-347.

Gurgul, A., E. Semik, K. Pawlina, T. Szmatoła, I. Jasielczuk and M. Bugno-Poniewierska (2014). "The application of genome-wide SNP genotyping methods in studies on livestock genomes." Journal of Applied Genetics **55**(2): 197-208.

Gómez, M. D., M. Valera, A. Molina, J. P. Gutiérrez and F. Goyache (2009). "Assessment of inbreeding depression for body measurements in Spanish Purebred (Andalusian) horses." Livestock Science **122**(2-3): 149-155.

Govaere, J. L. J., M. K. Hoogewijs, C. De Schauwer, J. Dewulf and A. De Kruif (2008). "Transvaginal ultrasound-guided aspiration of unilateral twin gestation in the mare." Equine Veterinary Journal **40**(5): 521-522.

Han, S. H., B. C. Yang, M. S. Ko, H. S. Oh and S. S. Lee (2010). "Length difference between equine ZFX and ZFY genes and its application for molecular sex determination." J Assist Reprod Genet **27**(12): 725-728.

Harper, J. C. and S. B. Sengupta (2012). "Preimplantation genetic diagnosis: State of the ART 2011." Human Genetics **131**(2): 175-186.

Hasegawa, T., F. Sato, N. Ishida, Y. Fukushima and H. Mukoyama (2000). "Sex Determination by Simultaneous Amplification of Equine SRY and Amelogenin Genes." Journal of Veterinary Medical Science **62**(10): 1109-1110.

Helszer, Z., A. Dmochowska, J. Szemraj, J. Slowikowska-Hilczer, M. Wieczorek, S. Jdrzejczyk and B. Kałuzewski (2013). "A novel mutation (c. 341A>G) in the SRY gene in a 46,XY female patient with gonadal dysgenesis." Gene **526**(2): 467-470.

Hinrichs, K., L. C. Buoen and G. R. Ruth (1999). "XX/XY chimerism and freemartinism in a female llama co-twin to a male." Journal of the American Veterinary Medical Association **215**(8): 1140-1141.

Hirota, K., T. Tozaki, S. Mashima and N. Miura (2001). "Cytogenetic assignment and genetic characterization of the horse microsatellites, TKY4-18, TKY20, TKY22-24, TKY30-41 derived from a cosmid library." Animal Genetics **32**(3): 160-162.

Holl, H. M., T. L. Lear, R. D. Nolen-Walston, J. Slack and S. A. Brooks (2013). "Detection of two equine trisomies using SNP-CGH." Mammalian Genome **24**(5-6): 252-256.

Hornak, M., M. Jeseta, P. Musilova, A. Pavlok, M. Kubelka, J. Motlik, J. Rubes and M. Anger (2011). "Frequency of aneuploidy related to age in porcine oocytes." PLoS ONE **6**(4).

Hughes, J. P. and A. T. Rommershausen-Smith (1977). "Infertility in the horse associated with chromosomal abnormalities." Australian Veterinary Journal **53**(6): 253-257.

Iannuzzi, L. and D. Di Berardino (2008). "Tools of the trade: diagnostics and research in domestic animal cytogenetics." J Appl Genet **49**(4): 357-366.

Iannuzzi, L., G. P. Di Meo, A. Perucatti, M. Spadetta, D. Incarnato, P. Parma, A. Iannuzzi, F. Ciotola, V. Peretti, G. Perrotta and R. Di Palo (2004). "Clinical, cytogenetic and molecular studies on sterile stallion and mare affected by XXY and sex reversal syndromes, respectively." Caryologia **57**(4): 400-404.

Juras, R., T. Raudsepp, P. J. Das, E. Conant and E. G. Cothran (2010). "XX/XY Blood Lymphocyte Chimerism in Heterosexual Dizygotic Twins from an American Bashkir Curly Horse. Case Report." Journal of Equine Veterinary Science **30**(10): 575-580.

Kakoi, H., K. Hirota, H. Gawahara, M. Kurosawa and M. Kuwajima (2005). "Genetic diagnosis of sex chromosome aberrations in horses based on parentage test by microsatellite DNA and analysis of X- and Y-linked markers." Equine Veterinary Journal **37**(2): 143-147.

Kakoi, H., T. Tozaki, K. Hirota, S. Mashima, M. Kurosawa and N. Miura (2000). "Ten equine microsatellite loci: TKY25, TKY26, TKY27, TKY28, TKY29, TKY267, TKY268, TKY269, TKY270 and TKY271." Animal Genetics **31**(1): 68-69.

Kalinowski, S. T., M. L. Taper and T. C. Marshall (2007). "Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment." Molecular Ecology **16**(5): 1099-1106.

Kent, M. G., R. N. Shoffner, A. Hunter, K. O. Elliston, W. Schroder, E. Tolley and S. S. Wachtel (1988). "XY Sex reversal syndrome in the mare: Clinical and behavioral studies, H-Y phenotype." Human Genetics **79**(4): 321-328.

Lacadena, J. R. (1996). Citogenética. Madrid, España.

Lear, T. L. and E. Bailey (2008). "Equine clinical cytogenetics: The past and future." Cytogenetic and Genome Research **120**(1-2): 42-49.

Lear, T. L., J. Lundquist, W. W. Zent, W. D. Fishback Jr and A. Clark (2008). "Three autosomal chromosome translocations associated with repeated early embryonic loss (REEL) in the domestic horse (Equus caballus)." Cytogenetic and Genome Research **120**(1-2): 117-122.

Lear, T. L. and R. B. McGee (2012). "Disorders of sexual development in the domestic horse, Equus caballus." Sexual Development **6**(1-3): 61-71.

McIlwraith, C. W., R. A. Owen and P. K. Basrur (1976). "An equine cryptorchid with testicular and ovarian tissues." Equine veterinary journal **8**(4): 156-160.

MacPherson, M. L. and J. M. Reimer (2000). "Twin reduction in the mare: Current options." Animal Reproduction Science **60-61**: 233-244.

Madill, S. (2002). "Reproductive considerations: mare and stallion." Vet Clin North Am Equine Pract **18**(3): 591-619.

Mäkinen, A., T. Hasegawa, M. Mäkilä and T. Katila (1999). "Infertility in two mares with XY and XXX sex chromosomes." Equine Veterinary Journal **31**(4): 346-349.

Mäkinen, A., L. Suojala, J. Niini, T. Katila, T. Tozak, Y. I. Miyake and T. Hasegawa (2001). "X chromosome detection in an XO mare using a human X paint probe, and PCR detection of SRY and amelogenin genes in 3 XY mares." Equine Veterinary Journal **33**(5): 527-530.

Martinez-Royo, A., E. Dervishi, J. L. Alabart, J. J. Jurado, J. Folch and J. H. Calvo (2009). "Freemartinism and FecXR allele determination in replacement ewes of the Rasa Aragonesa sheep breed by duplex PCR." Theriogenology **72**(8): 1148-1152.

Miller, A. and G. L. Woods (1988). "Diagnosis and correction of twin pregnancy in the mare." The Veterinary clinics of North America. Equine practice **4**(2): 215-220.

Miyake, Y. I., T. Inoue, H. Kanagawa, T. Ishikawa and K. Mogi (1982). "Chromosomal analysis and blood type examination of multiple births in equine." Japanese Journal of Veterinary Research **30**(1-2): 11-18.

Moreno-Millan, M., S. Demyda-Peyrás and D. Saleno (2012). "Sex chromosomes abnormalities in Purebred Spanish Horses with Reproductive Problems: Case Reports." Bull Univ Ag Sci Vet Med Cluj-Napoca **69**(1): 1-7.

Moreno-Millan, M., A. Rodero, F. J. Alonso and A. Sanz (1991). "First report of cytogenetic studies in Spanish breed horses." Genet. Sel. Evol **23**(SUPPL.): 176-178.

Morley, S. A. and J. A. Murray (2014). "Effects of Body Condition Score on the Reproductive Physiology of the Broodmare: A Review." Journal of Equine Veterinary Science **34**(7): 842-853.

Mucha, S., A. Wolc and T. Szwaczkowski (2012). "Bayesian and REML analysis of twinning and fertility in Thoroughbred horses." Livestock Science **144**(1-2): 82-88.

Munné, S. (2012). "Preimplantation genetic diagnosis for aneuploidy and translocations using array comparative genomic hybridization." Current Genomics **13**(6): 463-470.

Murray, J. D., C. Moran, M. P. Boland, A. M. Doff and C. D. Nancarrow (1985). "Cytogenetic analysis of 34 early stage bovine embryos from superovulated Hereford donors." Canadian Journal of Genetics and Cytology **27**(4): 483-486.

Osborne, E. C., A. O. Trounson and D. S. Cram (2005). "A QF-PCR system to detect chromosome 13 aneuploidy from as few as ten cells." American Journal of Medical Genetics **134 A**(1): 33-38.

Padula, A. M. (2005). "The freemartin syndrome: An update." Animal Reproduction Science **87**(1-2): 93-109.

Pailhoux, E., E. P. Cribiu, P. Parma and C. Cotinot (1995). "Molecular analysis of an XY mare with gonadal dysgenesis." Hereditas **122**(2): 109-112.

Peretti, V., F. Ciotola, S. Albarella, O. Paciello, C. Dario, V. Barbieri and L. Iannuzzi (2008). "XX/XY chimerism in cattle: Clinical and cytogenetic studies." Sexual Development **2**(1): 24-30.

Pieńkowska-Schelling, A., M. Bugno, M. Owczarek-Lipska, C. Schelling and E. Słota (2006). "Probe generated by Y chromosome microdissection is useful for analysing the sex chromosomes of the domestic horse." Journal of Animal and Feed Sciences **15**(2): 173-178.

Pinkel, D., T. Straume and J. W. Gray (1986). "Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization." Proceedings of the National Academy of Sciences of the United States of America **83**(9): 2934-2938.

Power, M. M. (1990). "Chromosomes of the horse." Adv. Vet. Sci. Comp. Med. **34**: 131-167.

Power, M. M. (1987). The Chromosomes of the Horse: Karyotype Definition, Clinical Applications and Comparative Interspecies Studies, University College Dublin.

Rambags, B. P. B., P. J. Krijtenburg, H. F. Van Drie, G. Lazzari, C. Galli, P. L. Pearson, B. Colenbrander and T. A. E. Stout (2005). "Numerical chromosomal abnormalities in equine embryos produced in vivo and in vitro." Molecular Reproduction and Development **72**(1): 77-87.

Raudsepp, T., A. Santani, B. Wallner, S. R. Kata, C. Ren, H. B. Zhang, J. E. Womack, L. C. Skow and B. P. Chowdhary (2004). "A detailed physical map of the horse Y chromosome." Proceedings of the National Academy of Sciences of the United States of America **101**(25): 9321-9326.

Raudsepp, T., K. Durkin, T. L. Lear, P. J. Das, F. Avila, P. Kachroo and B. P. Chowdhary (2010). "Molecular heterogeneity of XY sex reversal in horses." Anim Genet **41 Suppl 2**(SUPPL. 2): 41-52.

Révay, T., D. A. F. Villagómez, D. Brewer, T. Chenier and W. A. King (2012). "GTG mutation in the start codon of the androgen receptor gene in a family of horses with 64,XY disorder of sex development." Sexual Development **6**(1-3): 108-116.

Richer, C. L., M. M. Power, L. R. Klunder, R. A. McFeely and M. G. Kent (1990). "Standard karyotype of the domestic horse (Equus caballus). Committee for standardized karyotype of Equus caballus. The Second International Conference for Standardization of Domestic Animal Karyotypes, INRA, Jouy-en Josas, France, 22nd-26th May 1989." Hereditas **112**(3): 289-293.

Riera, F. L., J. E. Roldán, J. Gomez and K. Hinrichs (2016). "Factors affecting the efficiency of foal production in a commercial oocyte transfer program." Theriogenology **85**(6): 1053-1062.

Rodero Franganillo, A. and E. Rodero Serrano (2008). Las razas ganaderas de Andalucía. Sevilla, Junta de Andalucía.

Rodero-Serrano, E., S. Demyda-Peyrás, A. González-Martinez, A. Rodero-Franganillo and M. Moreno-Millán (2013). "The rob(1;29) chromosome translocation in endangered Andalusian cattle breeds." Livestock Science **158**(1-3): 32-39.

Rodríguez, I., J. Sanz and M. A. Failde (1992.). El caballo Español de estirpe Cartujana en América. Archivos de zootecnia. **41**.

Rosser, Z. H., P. Balaresque and M. A. Jobling (2009). "Gene Conversion between the X Chromosome and the Male-Specific Region of the Y Chromosome at a Translocation Hotspot." American Journal of Human Genetics **85**(1): 130-134.

Rowley, J. D. (1973). "A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining." Nature **243**(5405): 290-293.

Sato, F., K. Hirota, T. Tozaki, K. Ito, P. Dhakal, K. Taya, Y. Endo, H. Murase and Y. Nambo (2012). "A case of ambiguous external genitalia in a thoroughbred male horse with the 63,XO/64,XY Mosaic Karyotype." Journal of Veterinary Medical Science **74**(10): 1327-1331.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich (1988). "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase." Science **239**(4839): 487-491.

Sánchez-Guerrero, M. J., A. Molina, M. D. Gómez, F. Peña and M. Valera (2016). "Relationship between morphology and performance: Signature of mass-selection in Pura Raza Español horse." Livestock Science **185**: 148-155.

Scoggin, C. F. (2015). "Not just a number: Effect of age on fertility, pregnancy and offspring vigour in thoroughbred brood-mares." Reproduction, Fertility and Development **27**(6): 872-879.

Shinawi, M. and S. W. Cheung (2008). "The array CGH and its clinical applications." Drug Discovery Today **13**(17-18): 760-770.

Sinclair, A. H., P. Berta, M. S. Palmer, J. R. Hawkins, B. L. Griffiths, M. J. Smith, J. W. Foster, A. M. Frischauf, R. Lovell-Badge and P. N. Goodfellow (1990). "A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif." Nature **346**(6281): 240-244.

Slattery, J. P., L. Sanner-Wachter and S. J. O'Brien (2000). "Novel gene conversion between X-Y homologues located in the nonrecombining region of the Y chromosome in Felidae (Mammalia)." Proceedings of the National Academy of Sciences of the United States of America **97**(10): 5307-5312.

Sper, R. B., M. D. Whitacre, C. S. Bailey, A. J. Schramme, D. G. Orellana, C. K. Ast and J. M. Vasgaard (2012). "Successful reduction of a monozygotic equine twin pregnancy via transabdominal ultrasound-guided cardiac puncture." Equine Veterinary Education **24**(2): 55-59.

Sumner, A. T. (1972). "A simple technique for demonstrating centromeric heterochromatin." Experimental Cell Research **75**(1): 304-306.

Taberlet, P., S. Griffin, B. Goossens, S. Questiau, V. Manceau, N. Escaravage, L. P. Waits and J. Bouvet (1996). "Reliable genotyping of samples with very low DNA quantities using PCR." Nucleic Acids Research **24**(16): 3189-3194.

Taberlet, P. and G. Luikart (1999). "Non-invasive genetic sampling and individual identification." Biological Journal of the Linnean Society **68**(1): 41-55.

Tan, D. K. S. and N. Krekeler (2014). "Success rates of various techniques for reduction of twin pregnancy in mares." Journal of the American Veterinary Medical Association **245**(1): 70-78.

Thomsen, P. D. and P. H. Poulsenm (1993). "Analysis of the Gonadal Sex of Five Intersex Pigs Using Y Chromosomal Markers." Hereditas **119**(3): 205-207.

Treff, N. R. and R. T. Scott Jr (2012). "Methods for comprehensive chromosome screening of oocytes and embryos: Capabilities, limitations, and evidence of validity." Journal of Assisted Reproduction and Genetics **29**(5): 381-390.

Tozaki, T., N. Takezaki, T. Hasegawa, N. Ishida, M. Kurosawa, M. Tomita, N. Saitou and H. Mukoyama (2003). "Microsatellite Variation in Japanese and Asian Horses and Their Phylogenetic Relationship Using a European Horse Outgroup." Journal of Heredity **94**(5): 374-380.

Valera, M., A. Molina, J. P. Gutiérrez, J. Gómez and F. Goyache (2005). "Pedigree analysis in the Andalusian horse: Population structure, genetic variability and influence of the Carthusian strain." Livestock Production Science **95**(1-2): 57-66.

Van Der Schouw, Y. T., A. L. M. Verbeek and S. H. J. Ruijs (1995). "Guidelines for the assessment of new diagnostic tests." Investigative Radiology **30**(6): 334-340.

Van De Goor, L. H. P., H. Panneman and W. A. Van Haeringen (2010). "A proposal for standardization in forensic equine DNA typing: Allele nomenclature for 17 equine-specific STR loci." Animal Genetics **41**(2): 122-127.

Vandeplassche, M., L. Podliachouk and R. Beaud (1970). "Some aspects of twin-gestation in the mare." Canadian Journal of Comparative Medicine **34**(3): 218-226.

Villagómez, D. A. F., T. L. Lear, T. Chenier, S. Lee, R. B. McGee, J. Cahill, R. A. Foster, E. Reyes, E. St John and W. A. King (2011). "Equine disorders of sexual development in 17 mares including XX, SRY-negative, XY, SRY-negative and XY, SRY-positive genotypes." Sexual Development **5**(1): 16-25.

Villagómez, D. A. F., P. Parma, O. Radi, G. Di Meo, A. Pinton, L. Iannuzzi and W. A. King (2009). "Classical and molecular cytogenetics of disorders of sex development in domestic animals." Cytogenetic and Genome Research **126**(1-2): 110-131.

Villagómez, D. A. F. and A. Pinton (2008). "Chromosomal abnormalities, meiotic behavior and fertility in domestic animals." Cytogenetic and Genome Research **120**(1-2): 69-80.

Wade, C. M., E. Giulotto, S. Sigurdsson, M. Zoli, S. Gnerre, F. Imsland, T. L. Lear, D. L. Adelson, E. Bailey, R. R. Bellone, H. Blocker, O. Distl, R. C. Edgar, M. Garber, T. Leeb, E. Mauceli, J. N. MacLeod, M. C. Penedo, J. M. Raison, T. Sharpe, J. Vogel, L. Andersson, D. F. Antczak, T. Biagi, M. M. Binns, B. P. Chowdhary, S. J. Coleman, G. Della Valle, S. Fryc, G. Guerin, T. Hasegawa, E. W. Hill, J. Jurka, A. Kiialainen, G. Lindgren, J. Liu, E. Magnani, J. R. Mickelson, J. Murray, S. G. Nergadze, R. Onofrio, S. Pedroni, M. F. Piras, T. Raudsepp, M. Rocchi, K. H. Roed, O. A. Ryder, S. Searle, L. Skow, J. E. Swinburne, A. C. Syvanen, T. Tozaki, S. J. Valberg, M. Vaudin, J. R. White, M. C. Zody, E. S. Lander and K. Lindblad-Toh (2009). "Genome sequence, comparative analysis, and population genetics of the domestic horse." Science **326**(5954): 865-867.

Waits, L. P., G. Luikart and P. Taberlet (2001). "Estimating the probability of identity among genotypes in natural populations: Cautions and guidelines." Molecular Ecology **10**(1): 249-256.

Wagner, T., J. Wirth, J. Meyer, B. Zabel, M. Held, J. Zimmer, J. Pasantes, F. D. Bricarelli, J. Keutel, E. Hustert, U. Wolf, N. Tommerup, W. Schempp and G. Scherer (1994). "Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9." Cell **79**(6): 1111-1120.

Wallner, B., F. Piumi, G. Brem, M. Müller and R. Achmann (2004). "Isolation of Y Chromosome-specific Microsatellites in the Horse and Cross-species Amplification in the Genus Equus." Journal of Heredity **95**(2): 158-164.

Vodicka, R., R. Vrtel, O. Scheinost, J. Zapletalova, L. Dusek, G. Marie and J. Santavy (2004). "Refined quantitative fluorescent PCR of Y-chromosome DNA sequences mosaics in Turner's syndrome patients - Alternative to real-time PCR." Journal of Biochemical and Biophysical Methods **60**(2): 151-162.

Wolc, A., G. Torzynski and T. Szwaczkowski (2009). "Genetic effects on reproductive traits in Warmblood horses." Canadian Journal of Animal Science **89**(2): 215-218.

Wolfsdorf, K. E. (2006). "Management of Postfixation Twins in Mares." Veterinary Clinics of North America - Equine Practice **22**(3): 713-725.

Xiang, P. X., X. Dai, P. Leng, L. Liu and X. J. Hu (2013). "SRY gene-testing in the diagnosis of disorders of sex development among children." Chinese Journal of Contemporary Pediatrics **15**(7): 555-558.

PRODUCCIÓN CIENTÍFICA

PRODUCCIÓN CIENTÍFICA

Publicaciones en revistas indexadas en el *Journal of Citation Reports* de la Web of Science

AUTORES: Demyda-Peyrás S.; Membrillo A.; Bugno-Poniewierska M.; Pawlina K.; Anaya G.; Moreno-Millán M.
TÍTULO: The use of molecular and cytogenetic methods as a valuable tool in the detection of chromosomal abnormalities in horses: A Case of sex chromosome chimerism in a Spanish Purebred colt.
Revista: Cytogenetic and Genome Research. Año: 2013. Número: 141 (4). Páginas: 277-283.
ISSN: 1424:8581. DOI: http://dx.doi.org/10.1159/000351225
JCR Category: Genetics and Heredity. Position: 115/164. Impact Factor: 1,905 (Q3).

AUTORES: Demyda-Peyrás S.; Anaya G.; Bugno-Poniewierska, M.; Pawlina K.; Membrillo A.; Valera M.; Moreno-Millán M.
TÍTULO: The use of a novel combination of diagnostic molecular and cytogenetical approaches in horses with sexual karyotype abnormalities: A rare case with an abnormal cellular chimerism.
Revista: Theriogenology. Año: 2014. Número: 81(8). Páginas:1116-1122
ISSN: 0093-691X. DOI: http://dx.doi.org/10.1016/j.theriogenology.2014.01.040
JCR Category: Veterinary Sciences. Position: 17/129. Impact Factor: 1,845 (Q1).

AUTORES: Anaya G.; Moreno-Millán M.; Bugno-Poniewierska M.; Pawlina K.; Membrillo A.; Molina A.; Demyda-Peyrás S.
TÍTULO: Sex reversal syndrome in the horse: four new cases of feminization in individuals carrying a 64,XY SRY negative chromosomal complement.
Revista: Animal Reproduction Science. Año: 2014. Número: 151 Páginas: 22-27.
ISSN: 0378-4320. DOI: http://dx.doi.org/10.1016/j.anireprosci.2014.09.02
JCR Category: Agriculture, dairy & animal science – Position: 11/51. Impact Factor: (IF): 1,581 (Q1)

AUTORES: Anaya G.; Molina A.; Valera M.; Moreno-Millán M.; Azor P.; Peral P.; Demyda-Peyrás S.
TÍTULO: Sex chromosomal abnormalities associated with equine infertility: Validation of a simple molecular screening tool in the Purebred Spanish Horse.
Revista: Animal Genetics. Año: 2016. Número: Accepted in press
ISSN: 1365:2052. DOI: http://doi.org/10.1111/age.12543
JCR Category: Agriculture Dairy & Animal Science– Position: 8/58. Impact Factor: 1.779 (Q1).

AUTORES: Anaya G,; Valera M.; Molina A; Azor P; Moreno-Millán M.; Fernandez M. E.; Solé M.; Negro S.; y Demyda-Peyrás S.
TÍTULO: Blood chimerism in the horse is reproductively innocuous? a 15 cases report.
Animal Genetic, Sometido

Congresos Nacionales

AUTORES: Anaya G.
TÍTULO DE LA COMUNICACIÓN: Desarrollo de una herramienta molecular para la selección precoz de alteraciones reproductivas en caballos de pura raza española.
CONGRESO: III Congreso Científico de Investigadores en Formación en Agroalimentación ceiA3.
ENTIDAD ORGANIZADORA: Escuela Internacional de Doctorado en Agroalimentación eidA3.
TIPO DE PARTICIPACIÓN: Comunicación oral
PUBLICACIÓN: Libro de actas
LUGAR DE CELEBRACIÓN: Córdoba, España.
AÑO: 18 y 19 de Noviembre de 2014

Congresos Internacionales

AUTORES: Demyda-Peyrás S.; Anaya G.; Negro S.; Membrillo A.; Moreno-Millán M.
TÍTULO DE LA COMUNICACIÓN: Sex reversal syndrome in two Spanish Purebred mares: genetic and molecular studies
CONGRESO: 18th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR)
ENTIDAD ORGANIZADORA: ESDAR (European Society for Domestic Animal Reproduction)
TIPO DE PARTICIPACIÓN: Póster
PUBLICACIÓN: Reproduction in Domestic Animals 2014, 49 (3), 64-64.
ISSN**:** 0936-6768. DOI: . http://dx.doi.org/10.1111/rda.12391.
(IF): 1,392 (Q1). JCRC: Agriculture, dairy & animal science – Pos: 13/54
LUGAR DE CELEBRACIÓN: Helsinki, Finlandia
AÑO: 11 al 13 de Septiembre de 2014

AUTORES: Moreno-Millán M.; Anaya G.; Demyda-Peyrás S.
TÍTULO DE LA COMUNICACIÓN: Equine Infertility: genetic implications and their detection by molecular methodologies.
CONGRESO: The 14th International Symposium Prospects for the 3rd millenium agriculture
ENTIDAD ORGANIZADORA: USAMV(University of Agricultural Sciences and Veterinary Medicine)
TIPO DE PARTICIPACIÓN: Comunicación oral
PUBLICACIÓN: Libro de actas
LUGAR DE CELEBRACIÓN: Cluj-Napoca, Rumanía
AÑO: 24-26 de Septiembre de 2015

AUTORES: Anaya G.; Demyda-Peyrás S.; Moreno-Millán, M.
TÍTULO DE LA COMUNICACIÓN:
CONGRESO: Primer Congreso de la Sociedad Latinoamericana de Reproducción Animal.
ENTIDAD ORGANIZADORA: SOLARA (Sociedad Latinoamericana de Reproducción Animal)
TIPO DE PARTICIPACIÓN: Póster
PUBLICACIÓN: Libro de actas
LUGAR DE CELEBRACIÓN: Buenos Aires, Argentina
AÑO: 25-28 de marzo de 2015

AUTORES: Anaya G.; Moreno-Millán M.; Dorado J.; Valera M.; Peral P.; Molina A.; Demyda-Peyrás S.
TÍTULO DE LA COMUNICACIÓN: Molecular variability in 64,XY SRY negative intersex horses: preliminary results.
CONGRESO: 20th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR)
ENTIDAD ORGANIZADORA: ESDAR (European Society for Domestic Animal Reproduction)
TIPO DE PARTICIPACIÓN: Póster
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LUGAR DE CELEBRACIÓN: Lisboa, Portugal
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AUTORES: Anaya G.; Moreno-Millán M.; Bugno-Poniewierska M; Molina A.; Valera M.; Demyda-Peyrás S.
TÍTULO DE LA COMUNICACIÓN: Blood sex chromosomal chimerism is related to infertility in horses?: a six cases study.
CONGRESO: 18th International Congress on Animal Reproduction
ENTIDAD ORGANIZADORA: ICAR (International Congress on Animal Reproduction)
TIPO DE PARTICIPACIÓN: Póster
PUBLICACIÓN: Libro de actas
LUGAR DE CELEBRACIÓN: Tours, Francia
AÑO: 26 al 30 Junio de 2016

Patente

AUTORES: Anaya G.; Moreno-Millán M.; Membrillo A.; Molina A.; Valera M.; Demyda-Peyrás S.
TÍTULO: Método para el diagnóstico precoz de la infertilidad equina
NÚMERO: Patente española, P201630755
AÑO: 6 de Junio de 2016